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OBSERVATIONS ON THE METABOLISM OF *SARCINA LUTEA*. I

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The experiments reported in this communication were designed to provide some information on the dynamics of oxygen diffusion into living cells. It seemed desirable, *a priori*, to perform the observations on very small, spherical cells. To this end, micrococci were the forms chosen. After preliminary experimentation, it was found that a strain of *Sarcina lutea* which had long been maintained on artificial culture media fulfilled many of the requirements of the experiments: the organisms are very small and discrete (this strain no longer forms packets); the cells are spherical and highly uniform in diameter; and in water or dilute aqueous solutions they remain sufficiently viable for the requisite periods of time under the conditions used. As the experiments progressed there were noted a number of interesting characteristics of their metabolism, which are the main subjects of this paper.

METHODS

1. *Bacterial Cultures.*—(*a*) Preparation of suspensions: The organisms grow luxuriantly on plain nutrient agar prepared with peptone and fresh meat infusion or beef extract, with or without the addition of NaCl. After the completion of the preliminary experiments, the medium used contained one per cent Difco peptone, 0.35 per cent beef extract and 1.5 per cent agar in tap water. Agar slant cultures in tubes or Kolle flasks were grown for 20–24 hours at 37° C. The growth was washed off with distilled water, the suspension homogenized by gentle but prolonged shaking, the microörganisms precipitated by centrifugation and then washed by two suspensions in and

precipitation from distilled water. After the final washing, the sedimented cells were again suspended in distilled water and shaken to give a concentrated homogeneous suspension.

(*b*) Enumeration of cells and pH measurements: The numbers of bacteria per cc. were determined before and after exposure to the specific conditions of the experiments by direct microscopical observation and by plate count. The former was performed by the hemocytometer method, using a dilute solution of carbol fuchsin as a diluting fluid; the latter as commonly used in the enumeration of bacteria in water, milk and other fluids. The direct count includes all cells visible under the microscope and the plate count only viable cells which developed on the medium used into colonies visible to the eye aided by a 5–10 × hand lens. Although all reasonable efforts were made to insure accuracy, the usual presumptive errors of these methods apply to our data.

The suspensions used in our experiments were, before dilution, thick, yellow, pasty emulsions of the sedimented, packed cells. Microscopical observations indicate a high degree of freedom from detritus. The concordance of the direct and plate counts made before and after performance of the experiments implies a high coefficient of vitality and the essential absence of dead or senile cells in the suspensions when first prepared.

Measurements of hydrogen ion concentration were performed with indicators by a spot plate method. Inasmuch as the suspensions consist of the bacterial cells in distilled water or dilute aqueous solutions of low intrinsic buffering power, the stability of the pH of an exposed suspension is controlled primarily by the buffering powers of the cells. From preliminary titration experiments (after the method of Shaughnessy and Falk, 1924) it developed that the strain of *Sarcina lutea* used displayed rather slight buffering power. Hence, in performing colorimetric pH measurements, we determined with which of a series of solutions of very low buffer action, and varying but known pH values, the test solution was isohydric. The standard solutions were prepared by adding twice the usual concentration of indicator solution to water and adjusting the pH of 10 cc. samples to various levels (at 0.2 pH intervals) by adding appropriate quantities of HCl or NaOH (*cf.* Fawcett and Acree, 1929). The colorimetric standards were checked by electrometric measurements, using a buffer solution standard and the quinhydrone electrode. The results of several experiments appear in Table I.

It will be noted that the direct and plate counts generally agree closely at the outset of an experiment and are not too widely apart at

TABLE I

Hydrogen Ion Concentrations and Microbic Enumerations for Suspensions of Sarcina Lutea

Date 1929	Suspension	pH	Number of cocci per cc. ($\times 10^{-6}$)	
			Direct	Plate
Apr. 27	Original		3.2	1.7
	After 2 hours (room temperature):			
	Original		2.6	2.1
May 30	Original	7.4	143	140
	After 6 hours:			
	In water shaken in air	6.8	107	430
June 6	Original	7.4	63	64
	After 6 hours:			
	Original	7.2	150	30
	In glucose shaken in air	?	150	30
	In water shaken in N_2	7.6	32	27
	In glucose shaken in N_2	7.5	41	27
	In water shaken in air	8.0	40	7
July 5	Original		70	26
	After 6 hours:			
	Original		102	29
	In water shaken in air		23	9
	In glucose shaken in air		27	11
	In glucose shaken in 1% O_2		13	14
	In water shaken in 1% O_2		58	13
Aug. 7	Original		85	13
	After 11 hours:			
	Original	7.4	71	17
	In water shaken in air	7.8	23	8
	In glucose shaken in air	7.2	8	6
	In water shaken in N_2	7.2	6	8
	In glucose shaken in N_2	6.4	14	6
Oct. 16	Original	7.4	199	55
	After 26 hours:			
	In water shaken in N_2	7.8	182	29
	In glucose shaken in N_2	6.4	202	180
	In glucose shaken in air	6.0	139	35
	In water shaken in N_2	7.8	170	35
	In water shaken in air	7.6	148	32

the completion. When variations occur they are generally, as is to be anticipated, in the direction of lower plate than direct counts.

(*c*) Size of the bacteria: The diameters of the bacterial cells were determined by direct microscopical measurements with hanging drop preparations and a filar micrometer. There were no significant variations in the size of cells suspended in water and in dilute glucose suspensions, with or without traces of dilute, neutral fuchsin. The average diameter determined from several hundred measurements on the cocci was 1.275 micra. In this series the maximum was 1.42, the minimum 1.08. The distribution was approximately symmetrical about the mean and the probable error of the mean was $\pm$ 0.07 μ.

(*d*) Final handling: The thick bacterial paste obtained after centrifugation was diluted 1 : 1 with distilled water or with 1.0 or 0.5 per cent glucose in water and well mixed by shaking (with a glass bead). Of these suspensions 0.4 cc. (dry weight about 5 mgm., containing about 2×10^9 bacteria) or 0.8 cc. portions were placed in the manometer vessels for determination of oxygen consumption, etc., and a similar amount dried on a cover slip and weighed. In certain experiments oxygen was bubbled through the suspension for some time before beginning the observations. The pH was usually determined before and after the manipulation by the method described above.

2. *Gases.*—Tank nitrogen, of 99.7 per cent purity, was passed over heated copper and through glass connections directly into the manometers. Even this treatment did not give absolute freedom of oxygen, but traces that remained did not affect the results (see below). Gas mixtures containing 0.5 to 10 per cent oxygen were made by volumetric mixing of the treated nitrogen and air in large bottles over water. The nitrogen was always bubbled through first for a considerable time to remove dissolved air, and the mixture was used at once after preparation. To obtain accurate mixtures of 0.5 per cent or less, a manometric method was used. This is described below.

3. *Measurement of Oxygen Consumption, Carbon Dioxide Production, etc.*—The Warburg methods were used throughout. Small chambers of the cylindrical or conical type with an inset, used for studying metabolism of nerve (Gerard, 1927), were found highly satisfactory. With 0.4 cc. of bacterial suspension and 0.2 cc. of *N*/10 NaOH in the inset to absorb CO_2, the constants were either about 0.25 or 0.35, so that one cu. mm. change in gas volume gave a pressure difference of 3 or 4 mm. (of Brodie's solution). Oxygen consumption, Q_{O_2}, is expressed as cu. mm. consumed per hour per mgm. dry weight of the bacteria.

The respiratory quotient (R.Q.) was determined by comparison of the manometer readings when CO_2 was absorbed (NaOH in inset) and when allowed to accumulate (water in inset) in parallel experiments. Acid production was determined manometrically by the liberation of CO_2 from a bicarbonate-Ringer solution in the presence of 5 per cent CO_2 in nitrogen (Warburg, 1926).

All experiments were carried out at 20.1° C.

Gas mixtures with low concentrations of oxygen were obtained by mixing in a manometer. The Brodie solution used in the manometer capillary has a specific gravity so adjusted that 10,000 mm. equals 760 mm. of mercury. The pressure of the gas in the manometer chamber is easily obtained from the barometer reading and the difference of fluid level in the two limbs of the manometer. It is usually sufficiently accurate, in determining the percentage of oxygen, to assume 10,000 mm. Even the maximal correction for water vapor, if dry air is admitted, is only 5 per cent and may ordinarily be omitted. The oxygen admitted to the chamber filled with nitrogen is determined as follows: the fluid levels in the manometer are lowered (by a screw at the bottom) until there is a "negative" pressure in the chamber of *circa* 100 mm. (left side open to air = 50 mm., right side to chamber = 150 mm.). The chamber is then opened to the air for an instant by rapidly turning a stop-cock, the apparatus is shaken for a moment, the fluid level on the chamber side is brought to its initial setting and the level in the open limb read. This will now be, say, 200 mm. At constant volume and temperature then (the room air being nearly at the temperature of the thermostat and coming to rapid equilibrium) the gas pressure has been increased by 150 mm. of air or 30 mm. of oxygen. With a little practice it is possible to adjust the initial pressure difference and time of opening the chamber so that any desired oxygen percentage up to 0.5 can be obtained by one operation. This method permits the rapid and accurate preparation of gas mixtures with very low oxygen concentration and should be valuable in other studies where the influence of oxygen pressure on oxygen consumption is to be determined.

Results

1. *Respiration in Air.*—The rate of oxygen consumption for any sample of the bacteria used is not constant with time, but tends to be high at first and to fall rapidly at the outset and then slowly. Sometimes it remains at a constant low level from the third or fourth hour on; more often a slow fall continues through a 20-hour period. A typical curve is shown in Fig. 1, and the results of several experiments are given in Table II.

The initial level of oxygen consumption and the amount of fall have been fairly constant from one test suspension to another and variations are not related to variations in the ages of the cultures used. It might be anticipated that bacteria taken from a culture which has passed the period of logarithmic rate of growth would show an approxi-

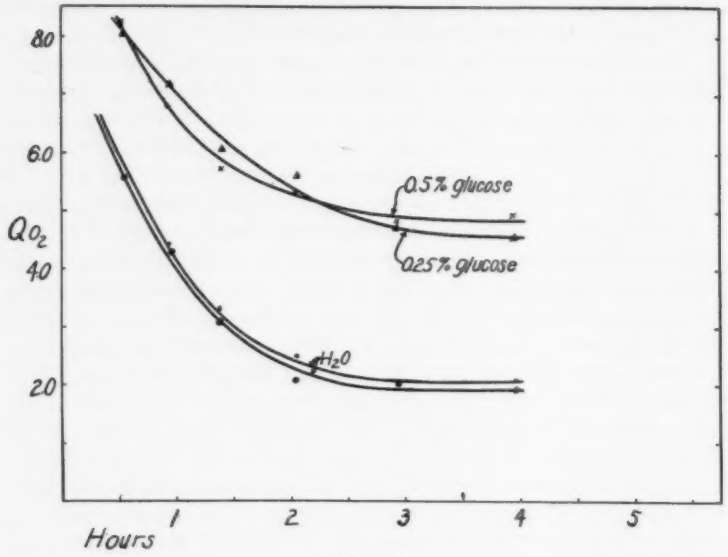

FIG. 1.

mately uniform level of oxygen consumption. Although viability generally decreases in the non-nutrient menstrua, the rate of this process is far too low to be invoked as the explanation of the decline in oxygen consumption. The question arises: In what way does the preparation of the suspension and its treatment in the manometer chamber lead to the establishment of a "time zero"? In other words, if the oxygen consumption of these cocci is assumed to have been essentially constant in time, what causes it to begin a rapid fall when its measurement is undertaken?

If a progressing mortality of the individual cells does not seem to explain the findings, the early high respiration cannot, on the same evidence (some variable fall in plate counts from start to end), be interpreted as due to continued growth which was later suspended. Continued agitation, gentle or vigorous, has been shown to have no effect; and temperature and suspension menstruum can also be

TABLE II

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
May 14	Water suspension, in air	6.0	3.5	2.3		1.6	
	Water suspension, in air	6.5	3.5	2.3		1.7	
	0.2% glucose suspension, in air		5.5	3.8		2.4	
	0.2% glucose suspension, in air		5.5	3.4		2.2	
May 30	Water suspension, in O_2	4.4	2.2		2.0		
	Water suspension, in air	4.3	2.0		2.0		
	0.5% glucose suspension, in air	6.9	5.1		5.0		
	0.2% glucose suspension, in air	7.1	5.2		4.6		
June 6	Water suspension, in air		6.8	5.2	4.0	3.3	2.8
	Water suspension, in 1% O_2		2.9	2.3	2.0	1.8	1.6
	0.2% glucose suspension, in 1% O_2		5.0	3.7	3.0	2.6	2.3
July 3	Water suspension, in 2.5% O_2	5.2	2.7	1.9	2.1	1.7	
	Water suspension, in 1% O_2	6.0	2.7	2.1	2.2	1.7	
	0.2% glucose suspension, in air	7.2	5.2	4.5	4.4	3.7	
	0.2% glucose suspension, in 2.5% O_2	11.0	5.1	4.0	3.7	3.1	

TABLE II—*Continued*

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
Aug. 7	Water suspension, in air	8.6		← 4.1 →			
	Water suspension, in air after 4 hours anoxia	14.4		6.2			
	Water suspension, in air after 4 hours anoxia	11.8		6.0			
	0.2% glucose suspension, in air	21.1		11.6			
	0.2% glucose suspension, in air after 4 hours anoxia	34.8		19.0			
	0.2% glucose suspension, in air after 4 hours anoxia	31.8		16.6			
Oct. 16	Water suspension, in air	3.2	2.1	1.8		←1.2→	1.1 (9 hrs.)
	Water suspension, in air after 1 hour N_2 bubbling	3.3	←	1.7 →		←	1.1 → (9 hrs.)
	Water suspension, in air after 1 hour O_2 bubbling	1.8	←	1.4 →		←	1.1 → (9 hrs.)
	Water suspension, in air after 13 hours anoxia	9.1		2.1			
	0.2% glucose suspension, in air after 1 hour anoxia	7.5	←	3.8 →		←	2.1 → (9 hrs).
	0.2% glucose suspension, in air after 13 hours anoxia	12.2		4.6			
Nov. 14	Water suspension, in air	4.3	←3.1→		←1.7→		←1.3→ (9 hrs.)
	Same	4.3	←2.5→		←1.7→		←1.3→ (9 hrs.)
	0.2% glucose suspension, in air	9.3	←3.3→		←1.8→		←1.6→ (9 hrs.)
	Same	8.8	←4.5→		←2.0→		←1.6→ (9 hrs.)

excluded as factors leading to the fall in oxygen consumption. It might then be assumed that the initial values are excessive, probably largely as a result of partial asphyxia of the organisms induced in the course of their preparation (and partly perhaps due to food reserves stored from the nutrient medium), and that the lower values obtained some time after an experiment is begun are to be regarded as the "normal" ones rather than as "abnormal" levels resulting from some depression. It may be noted that Callow (1924) obtained a Q_{O_2} of 7.0, constant for 10 hours, using *Sarcina aurantiaca.*

When the organisms are suspended in a 0.2–0.5 per cent glucose solution instead of water, the same progressive fall of oxygen consumption is observed. The Q_{O_2} values at each point in time are, however, much greater in the former case (by 100 per cent to 300 per cent), so that the respiration curve for the bacteria in the glucose-containing menstruum lies above but parallel to that for the bacteria in water. (Fig. 1, Table II.) The influences of glucose and other substances on the respiration of *Sarcina lutea* are reported upon in more detail in the following paper.

The R.Q. in water and in glucose solution was determined in two experiments. In water it remained constant at about 0.67 during 8 hours. (Gotschlich, 1912, cited quotients of 0.71–0.78 for bacteria metabolizing in the absence of fermentable substances, and Stephenson and Whetham, 1923, found R.Q.'s of about 0.7 after all added glucose had been utilized.) In glucose solution, the R.Q. for the first hour was 0.95 and then fell during seven hours to 0.71, the average for the whole run being 0.81. These figures suggest that, even in glucose solution, less than half the oxygen consumed is used to oxidize carbohydrate, although the presence of glucose has more than doubled the amount of oxygen taken up. (These values of R.Q. are lower than those recorded for bacteria on a nutrient pabulum capable of supporting their growth. *Vide* Soule, 1928.)

2. *The Influence of Oxygen Pressure.*—The oxygen consumption of *Sarcina lutea* in equilibrium with oxygen at varying partial pressures has been determined and it has been found that in air or pure oxygen the respiration is essentially the same. When the bacteria were held in water, with a lower rate of respiration, 2.5 per cent oxygen in the gas or even one per cent oxygen, in two cases, sufficed as well as the pure gas. With the glucose menstruum (and more rapid oxygen consumption) one per cent oxygen was definitely too low a concentration to support full respiration and 2.5 per cent of the gas was sometimes inadequate. Concentrations below one per cent were uniformly insufficient to support the rate of oxygen consumption possible in air.

When, after a period of complete anoxia, oxygen was admitted to a concentration of 0.5 per cent or less, there was at once a moderate oxygen consumption. Although the rate of respiration was already far less than in air, it fell off considerably in a short time, much as previously described for experiments in air (Fig. 1). The fall in this case was not due to a diminishing supply of oxygen following utilization of the small amount admitted, for it occurred when only 5 to 10 per cent of the oxygen had disappeared. More than doubling the amount of shaking did not affect the value of the critical oxygen pressure That failure to attain equilibrium between gas and liquid phases did not play a rôle is further attested by experiments made in another connection. Hydrogen was absorbed by an unsaturated fat in water suspension under the influence of a catalyst at a rate over 100 times that of the oxygen absorption here, yet the rate of shaking was adequate.

3. *Oxygen Debt.*—It seemed possible that the regular occurrence of a relatively high oxygen consumption directly after admitting this gas, or at the start of an experiment as the bacterial suspensions became well oxygenated by shaking, might represent the discharge of an oxygen debt. That such a debt can be accumulated is easily demonstrated: a suspension kept for some hours in nitrogen and

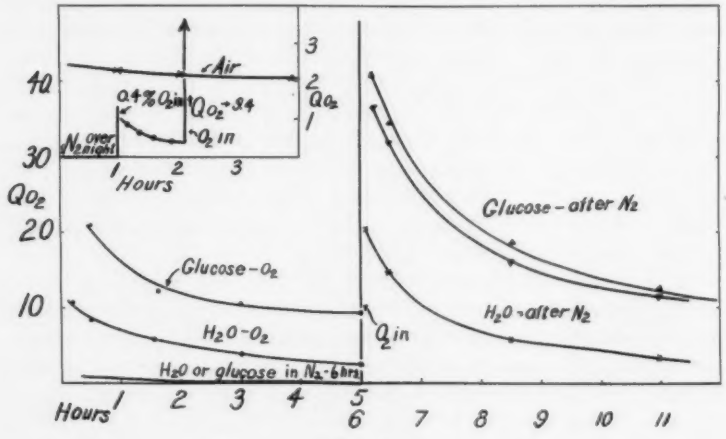

FIG. 2.

then exposed to air takes up oxygen at a greatly increased rate (Fig. 2). At the start, this rate may be considerably greater than observed when the bacteria are kept in air throughout. It subsequently

decreases just as under the conditions previously considered. This burst of increased oxygen consumption is greater after long than after short periods of anoxia, and for the same period is greater when the bacteria are suspended in glucose solution than in water (Table II). Glucose is capable, apparently, of stimulating the metabolism of these organisms not only in the presence of oxygen (leading to a more rapid consumption of the gas), but also in its absence (leading to an increased oxygen debt).

To determine whether previous anoxia was the cause of the high respiration observed at the start of the simple experiment in air, the following test was made. A particular bacterial suspension was divided into two portions. Oxygen was bubbled through one and nitrogen through the other for an hour and then each was rapidly introduced into manometers in air. The usual high initial oxygen consumption values were obtained with the sample treated with nitrogen, while they were much lower for the oxygen-treated portion. The slow fall, continuing for many hours after mounting, occurred with both. This slow fall of oxygen consumption with time might be related to the exhaustion of the substrate to be oxidized rather than to the oxygen itself. In the case of water suspensions, at least, the only material for oxidation that the cells have is their own substance and such food as may be stored during growth in a nutrient medium. During 24 hours the carbon in the carbon dioxide given off corresponds to 1 to 2 per cent of the dry weight of the organisms.

4. *Acid Formation.*—The oxygen debt these cells incur in an atmosphere of nitrogen might be due to accumulation of oxidizable metabolites or to exhaustion of an oxidizing reserve. The first case is reminiscent of the metabolism of muscle and the rôle played by lactic acid, the second suggests the metabolism of nerve. We therefore attempted to ascertain whether acid is produced in the metabolism of the microbic cells under the conditions of our experiments—especially in the absence of oxygen.

The pH of the cell suspension was determined in many experiments before and after manipulation in the manometers, using the indicator method described above. There was no consistent change under any of the conditions studied—water or glucose solution, oxygen present or absent. The initial and final pH values were about 7.4, though occasional final values were as high as 8.0 and as low as 6.0, especially in glucose suspensions. Acid formation was also measured manometrically by the liberation of CO_2 from bicarbonate-Ringer solution in equilibrium with 5 per cent CO_2 in N_2. This was studied in the presence or absence of glucose. During eight hours' asphyxia, the

CO_2 liberated, if due to lactic acid formation, indicated a production of 0.0023 mgm. of lactic acid per mgm. dry weight per hour in glucose solution and 0.0001 mgm. lactic acid in water (one experiment each). The quantities of lactic acid formed in eight hours would require, respectively, 14.5 and 0.6 cu. mm. of O_2 for complete oxidation. For many cells, including bacteria, studied by Meyerhof (1927) (also: Meyerhof and Finkle, 1925) and others, the oxidation quotient (the lactic acid produced in nitrogen minus that formed in oxygen, divided by the oxygen consumed) is 3 or more. That is, one equivalent of oxygen consumed prevents the appearance of three times as many equivalents of lactic acid as it could oxidize. In these experiments, in eight hours' exposure in an oxygen atmosphere, the bacteria consumed: in glucose solution, 24 cu. mm., and in water, 17.5 cu. mm., of oxygen. The oxidation quotients are, therefore, 0.6 and 0.03. The lactic acid (?) produced during asphyxia was only one-fifth of what might have been anticipated in the glucose solution and was practically zero in the water.

These results may be expressed in another way. In other experiments, in either water or glucose, following a six-hour asphyxia the average extra oxygen taken up during 6 hours was 50 per cent of the normal oxygen consumed by the same number of bacteria in the same time. The total oxygen debt in these experiments may therefore be assumed to have been 9 cu. mm. in glucose solution and 5 cu. mm. in water. Since in the water suspension only 0.6 cu. mm. of oxygen could have been used to oxidize acid metabolites (typified by lactic acid), nine-tenths or more must have acted in some other manner. In glucose solution, the extra oxygen might have been used entirely to oxidize metabolites, though presumably here also some would be used otherwise. The possibility must be recognized, of course, that more acid may have been formed than is recorded by the CO_2 liberation, some being neutralized by other cell buffers. Also, intermediate substances of too weak acidity to result in the liberation of CO_2 would escape detection by the methods used.

5. *Oxidizing Reserve.*—The existence of a large oxygen debt after asphyxiation, with no demonstrable accumulation of incompletely oxidized metabolites to account for it, suggests that the extra oxygen is utilized to replenish an oxidizing reserve which had been drawn upon to continue oxidations during asphyxia. If this were the case, some CO_2 should appear during the asphyxial period and a correspondingly smaller amount during the following oxygen period. The R.Q. of the extra respiration, while the oxygen store is being refilled, should be correspondingly low. This does not appear to be the case,

for the R.Q. of suspensions in water is the same early or later after commencing a series of observations, whether or not the extra oxygen consumption is occurring. Also, the absence of CO_2 liberation in nitrogen from water-bicarbonate suspensions renders improbable the production of CO_2 as well as of non-volatile acids. We do not wish, on the basis of the few experiments performed, to conclude finally that the oxygen debt developed by *Sarcina lutea* is due to the accumulation of non-acid metabolites and not to the depletion of oxidizing reserves, though this seems most probable. The work unfortunately could not be continued at the time and the present report must suffice to record these preliminary observations. It is interesting to note that Meyerhof (1912) found in some of his studies on *Vibrio metchnikovi* that in the absence of oxygen all chemical activity and energy liberation appeared to be simply suspended. The falling Q_{O_2} with time observed for *Sarcina*, in contrast to the constant respiration of bacteria observed by Callow (1924), may be related to such differences.

Summary

The respiration of a strain of *Sarcina lutea*, growing as individual cocci of uniform size and of relatively high viability under our experimental conditions, was studied by means of the Warburg technique. Suspensions of thoroughly washed bacteria in water or in glucose solution were enumerated by direct and plate counts, and pH determinations were made before and after manipulation in the manometer. A simple method is described for obtaining accurate gas mixtures with a low concentration of oxygen.

The oxygen consumption of various suspensions of the washed cocci was found to be fairly constant in water suspension at about 2.6 cu. mm. O_2 per mgm. dry weight per hour, or approximately 7 μ^3 O_2 per single cell per hour: that is, over three times its volume of oxygen was consumed per hour by each micrococcus. This value is for the nearly constant level attained some hours after the start of the experiment. Oxygen consumption is considerably more rapid at first and falls along a roughly arithlogarithmic curve towards an asymptote. This early excess appears to represent an oxygen debt due to partial asphyxia produced in the course of preparation of the suspensions.

In 0.2 to 0.5 per cent glucose solution, the rate of oxygen consumption falls along a similar curve, but the rates are 100 to 300 per cent greater than for suspensions of cocci in water.

The respiratory quotient in water is constant at about 0.67; in glucose it falls from 0.95 to 0.71 in seven hours (one experiment each).

The oxygen consumed by a suspension in water is independent of oxygen concentration when this is above one per cent. For glucose suspensions 2.5 per cent O_2 or more is required for maximal consumption. Below the critical values of oxygen tension, oxygen consumption becomes less with diminishing oxygen concentration. When air is admitted to the suspension after a period of complete anoxia, there is observed a high initial rate of consumption followed by progressively declining rates during several hours until a relatively constant rate is approached. When small amounts of oxygen (0.5 per cent or less) are admitted after anoxia, consumption is greater at first and then falls as when air is admitted; but the maximal rate is low, usually less than the normal in air.

The oxygen debt developed by these organisms is not apparently associated with the production of detectable acid metabolites (in water suspensions, at least), nor with the formation of carbon dioxide during asphyxia. It may result from the accumulation of non-acid metabolites, or of amounts of acid metabolites that do not equal or exceed the buffering capacities of the cells. The oxidation quotient, even in glucose solution, in contrast to the data for most cells that have been studied, is not over 0.6.

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OBSERVATIONS ON THE METABOLISM OF *SARCINA LUTEA*. II

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In the preceding paper the effects of anærobiosis on this organism and the influence of glucose on its oxygen consumption have been reported. It seemed desirable to extend these observations to the influence of lactic acid, as one of the carbohydrate intermediates, and also to study the influence of the respiratory catalyst, methylene blue, and of the inhibitors, carbon monoxide and cyanide. The results of these experiments have been surprising in several respects. The methods used were exactly as previously described, and all experiments were carried out at 22° C.

RESULTS

1. *Water and Saline Suspensions.*—This series of 34 experiments in water shows an average Q_{O_2} of 2.5 during the relatively steady period five hours after the start of readings (Table I). This is in good

TABLE I

Q_{O_2} in Water Suspension

Initial value Q_{O_2}	Later value	
	Hours	Q_{O_2}
8.1 (4)*	5	3.5
9.3 (4)	5	3.3
7.2 (4)	4.5	2.4
7.5 (4)	4 +	2.5
8.5 (2)	4.5	1.8
5.3 (4)	5	2.1
5.8 (2)	4.5	2.0
5.1 (3)	3.5	2.2
4.7 (2)	6	1.2
6.1 (1)	5.5	3.0
8.0 (4)	5	2.3
? 1.4 (2)	5	0.8
? 1.8 (3)	3	1.2

* Bracketed number represents number of separate runs.

agreement with the value of 2.6 for the first, smaller series. The temperature was slightly higher in this series but the average was

taken at a somewhat later time. The initial Q_{O_2} average is 7.1, a value depending on the conditions of preparation, as previously discussed. Two experiments at 37° C. compared with 22° C. gave a Q_{10} of approximately 1.7.

Barron and Harrop (1929) found a marked effect of crowding on the Q_{O_2} of white blood cells, the respiration per unit of material falling as more and more concentrated suspensions were used. The possibility of a similar effect in these experiments was tested by varying the density of the suspension from the start or by suddenly diluting a water suspension during a run by tipping in more water. No change was observed, so that this factor may be excluded. It might well depend, in the case of the blood cells, on limitation of motility of the undulating membrane in the heavier suspensions.

The effect of NaCl in concentrations up to 1.1 per cent was determined, as a control of osmotic and ion effects with other reagents. The lower concentrations were without effect, and even the highest used gave a maximum decrease of 20 per cent of the Q_{O_2} as compared with water, and this only occasionally. Further addition of one part to ten of M/15 phosphate buffer was likewise of no consequence for the oxygen consumption; nor was the pH, between 7 and 8. When salt solutions were tipped into a water suspension of respiring bacteria, a small brief acceleration was often observed, after which the original curve was resumed (Fig. 3). Whether this is a true momentary "stimulation" or some small experimental error has not been further investigated; in either case the results serve as a control for the observed changes when other substances are tipped in.

2. *Methylene Blue.*—Addition of methylene blue (0.2 per cent or 0.1 per cent methylene blue "for vital staining") to a water or buffered saline suspension always raised the Q_{O_2} by about one hundred per cent; from an average value of 1.9 to one of 3.9 in seven experiments, an increase of two. The increased oxygen consumption tends to fall off with time but remains above that of water for several hours. The effect of methylene blue plus glucose, sodium lactate, or sodium cyanide will be considered with those substances.

3. *Glucose.*—The effect of glucose addition has been followed somewhat further than in the preceding paper, by tipping a glucose solution into a water suspension of the cocci during a run. This makes it possible to obtain the entire glucose effect, since no time is lost after its addition, during which readings cannot be made. Table II sums up all the results with plain glucose. The average Q_{O_2} of 2.2 before tipping in the sugar rose to 10.5 for the first period (one-half to one hour) after tipping and then fell. At three and one-half

TABLE II

Respiration in water before addition	Added		Maximum after addition	Later value	
	Glucose	Methylene blue			
Q_{O_2}	Per cent		Q_{O_2}	Hours	Q_{O_2}
* 2.3	.5	+	13.6	6	1.3
* 2.5	.5	−	10.4	6	4.1
2.1	8.8	−	15.0	5	4.5
2.0	0.4	−	9.7	4.5	4.5
1.9	0.2	−	9.3	2	8.5
2.5	0.8	+	14.6	2	14.4
2.3	0.8	−	9.2	2	9.7
2.2	0.2	+	19.2	2 (18)	4.9 (0.5)
2.3	0.2	−	10.0	2 (18)	4.4 (1.2)
2.3	0.8	+	18.2	2 (18)	13.2 (0.3)
2.3	0.8	−	12.2	2 (18)	8.2 (0.5)

* Parallel runs.

hours the average Q_{O_2} was six. The maximum reached was independent of glucose concentration (though 8.8 per cent glucose gave the greatest increase observed), but the subsequent fall was somewhat slower with larger amounts than smaller.

The total excess oxygen consumed by the bacteria due to the addition of glucose can be determined by subtracting the Q_{O_2} curve in water from that with glucose added. In at least two cases this has been definitely greater than the amount required to completely oxidize all the added glucose, which suggests a "specific dynamic action" of this substance. Confirmatory evidence is obtained from the R.Q. findings, discussed previously, and from the further observation that suspensions carried for 24 hours in glucose solutions have a lower Q_{O_2} than similar ones in water, as if the higher rates at first had more completely exhausted the cell reserves. Evidence of a stimulating action of glucose in nitrogen has also been previously presented. All these observations indicate a dynamic action of glucose though they are hardly extensive enough to permit sweeping conclusions.

Methylene blue plus glucose leads to a greater initial increase of oxygen consumption than the sum of their separate effects, but this excess becomes less with time (Fig. 1). Thus, in four parallel experi-

ments, the average maximum in glucose was 11.2, in glucose plus 0.2 per cent methylene blue, 16.5. Seven hours later the values were 4.7 and 5.8, and the next morning, in all cases followed, the values were higher in the suspensions in glucose without the dye. This diminishing or reversing of the methylene blue increase with time is

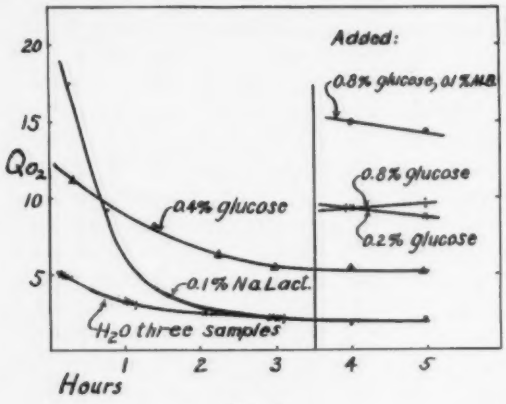

FIG. 1.

similar to the results with water soiutions of the dye or of glucose alone. The absolute increase of Q_{O_2} when methylene blue is added to water suspensions is 2.0, but the percentage increase is about one hundred. When the dye is added to glucose suspensions, the absolute increase is over 5, the percentage increase fifty. The interpretation comes to mind that these two substances act at successive steps in the chain of oxidative reactions, thus leading to a product of their separate effects, rather than concomitantly, which should give only the sum. Much evidence exists that this is indeed the situation. The recent findings of Warburg, Kubowitz and Christian (1930) and Wendel (1930) indicate that reduction of methylene blue may lead to peroxide formation with cytochrome, which in turn oxidizes other substances (as lactic acid to pyruvic acid), and leaves the iron in the Fe^{+++} stage. The second reaction inight well depend, if only indirectly, on the amount of oxidizable material (glucose) present. The findings with lactate additions, however, are not entirely in harmony with this view.

4. *Sodium Lactate.*—The greatest rates of oxygen consumption obtained resulted from the addition of lactate to water or saline suspensions. The maximum Q_{O_2} after tipping averaged over ten times that just before, and a more than twenty-fold increase has been observed. Though the maximum Q_{O_2} varied in different runs from

17 to over 50, the variation was low in any one run and not related to the concentration of lactate added (Fig. 2). (In one experiment a regular rise in maximum with concentration was observed.) The subsequent course of the extra oxygen consumption, on the other

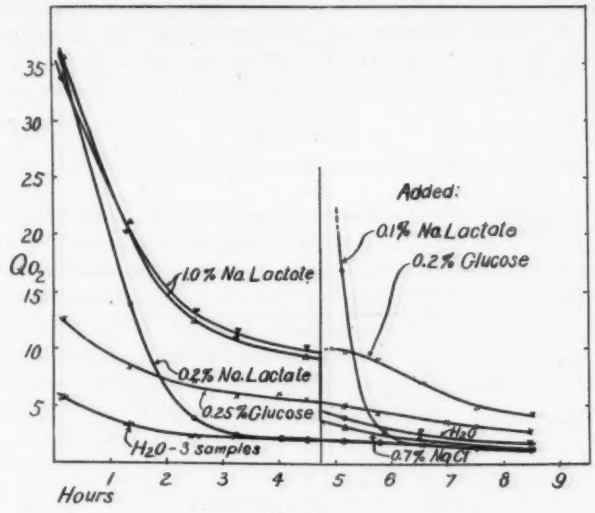

FIG. 2.

hand, was intimately dependent on this concentration (Figs. 2 and 3, Table III). Thus, the average of a series of twelve experiments gave a Q_{O_2} of 2 in water, and a maximum of 23 after sodium lactate was tipped. Five hours later the Q_{O_2} values for various concentrations were:

0.05% = 1.4; 0.1% = 2.5; 0.25% = 3.0; 0.55% = 6.0; 1.1% = 7.0.

Such a falling off with time, of the lactate effect, is due in part to the actual removal of lactate, but cannot be accounted for entirely on this basis. A few hours after the addition of 1.0 per cent lactate ions, the Q_{O_2} will have fallen to, say, one-half of the maximum. It can be inferred (see below) with considerable certainty that over 0.5 per cent lactate is still present. It has been found, however, that when 0.5 per cent lactate is freshly added to a water suspension, the Q_{O_2} reaches the same maximum as when 1.0 per cent is added; so that the falling off in the first case must be complicated by other factors. An occasional finding, which also indicates the complexity of the system, is an immediate increase of respiration rate after lactate

(or glucose) addition followed by a further rise for an hour or more before the usual fall sets in. This appeared oftener with the more concentrated additions, as if the strong lactate partly inhibited respiration until some was metabolized away.

The amount of lactic acid actually burned may be estimated from the excess oxygen consumed, on the assumption that just this extra oxygen is all used to completely oxidize the acid. In most experiments 0.2 cc. of a 0.2 per cent solution of lactate (as the sodium salt)

TABLE III

Respiration in water before addition	Added		Maximum after addition	Later value	
	Sodium lactate	Other substance			
Q_{O_2}	Per cent		Q_{O_2}	Hours	Q_{O_2}
3.5	.2	Methylene blue	48	7	1.5
4.3	.2	Methylene blue	52	7	0.5
4.5	.2	—	65	7	3.0
2.6	.2	Methylene blue	17	6	1.1
2.5	.2	—	20	6	1.9
1.8	2.2	In water	17	4.5	8.3
	1.1	In NaCl, PO_4 buffer, pH7	18	4.5	7.4
1.4	1.4	Same, pH8	19	4.5	8.2
2.2	2.2	—	29	5	5.5
2.0	0.5	—	28	5	3.0

was tipped into 0.2 cc. of the bacterial suspension, giving a final concentration of 0.1 per cent. This quantity of lactic acid, 0.0044 millimols, would require 298 cu. mm. of oxygen for full oxidization. The values actually obtained for the extra oxygen consumed (the area under the Q_{O_2} curve with lactate present less that with lactate absent —the two curves usually joining some hours after the start) were:

210, 250, 200, 270, 310.

The average, 250, is somewhat less (15 per cent) than theoretical for the complete burning of all the lactate. This is probably a technical error due to failure to rinse all the fluid from the side bulb into the main chamber, with the consequent exclusion of some of the lactate from the bacteria. Unfortunately, the quantitative possibilities were not in mind when the experiments were carried out, and the usual to and fro pouring was ordinarily omitted because of the danger of

spilling alkali—a narrow opening to the side-bulb made rather severe tapping necessary in some cases. In general, the agreement is good enough to indicate complete oxidation of small amounts of lactic acid.

With larger quantities, that is, more concentrated solutions, oxidation was not complete even in twenty-four hours—when the extra oxygen consumption had ended. Thus for a 1.0 per cent solution 1500 cu. mm. of oxygen would have been required for full oxidation. The excess oxygen totalled over 700 cu. mm., and the total oxygen used (*i.e.*, the lactate Q_{O_2} curve not corrected by the water control) after the lactate addition only about 900 cubic millimeters.

The possibility at once suggests itself that the falling off of the excess respiration, and the failure of complete oxidation of the larger amounts of lactic acid are alike due to the accumulation of injurious reaction products. A rise in pH, in particular, must result from the conversion of lactate ion into CO_2, two-thirds of which would leave the bacterial suspension and be absorbed in strong alkali in the inset of the vessel, while one-third would remain to combine with the free Na^+ ions, giving $NaHCO_3$ in place of $NaC_3H_5O_3$. Such a pH change is not, however, a critical factor; for suspensions buffered at pH 7 or 8 show the same Q_{O_2} curve with added buffered sodium lactate as do

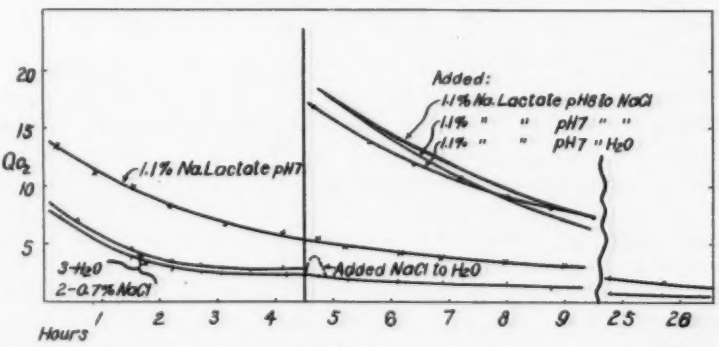

FIG. 3.

water suspensions (Fig. 3, Table III). Also, in one series, a phosphate buffer at pH 5.9 was added to the suspensions in 1/10 and 1/5 concentrations. After a long run with much added lactate, the 1/10 buffer was exhausted and the suspension alkaline to litmus, while that with the stronger buffer was still faintly acid. The Q_{O_2} curves, however, were almost perfect duplicates. Further, it is doubtful if any kind of inhibiting end-products can be involved. If such were the case,

dilution of a suspension which has had lactate added some time before should cause the fallen Q_{O_2} to again rise to some extent. Actually the reverse is found; dilution promptly lowers the Q_{O_2} still further (Fig. 2).

Finally, it is interesting to note the effect of a double addition of lactate (Fig. 4). A suspension in 0.3 per cent lactate at an early stage (1–2 hours) and with a markedly increased respiration is in no way affected by the further addition of one-third its volume of 0.6

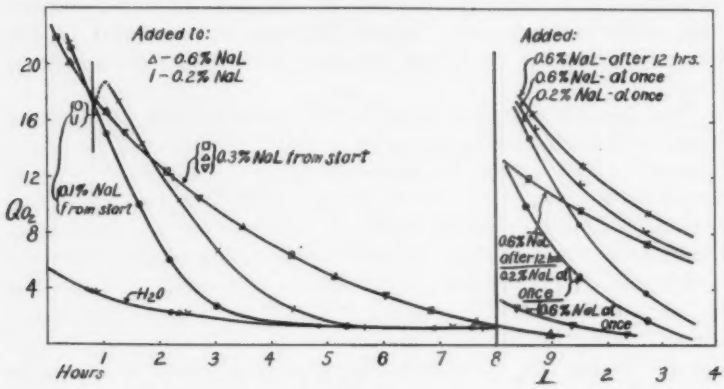

FIG. 4.

per cent lactate. There is no immediate increased oxygen consumption nor any delay in the subsequent fall. If the second portion of lactate is added at the end of nine hours, when the original lactate action has ended and respiration is the same as in a water control, there is some increased consumption but much less than in the control. In one experiment, for example, the Q_{O_2} in both water and lactate suspensions had fallen to 1.5. When new lactate was added to the lactate suspension, this value rose only to 3, while the Q_{O_2} of the water suspension rose to eighteen. If the lactate addition is delayed another 12 hours, however, the water and lactate suspensions being shaken in the usual manner in manometers, the difference is much less marked. Thus, in the same set of runs, lactate added to the water suspension 21 hours after the start again gave a Q_{O_2} of 18, and added to the lactate suspension, one of 12 instead of three. Obviously, a second lactate addition has no effect when added soon after a first one, some effect when added after the initial increase in respiration is past, and close to a maximal effect when added the following day.

When the experiment is carried out with 0.1 per cent lactate and 0.2 per cent lactate added later, the time relations are altered. With

these dilutions, a second addition has some effect even two hours after the first. Thus, in one case (Fig. 4), the fall in respiration was delayed an hour or more and some actual increase may have been present at once after the second addition. It is interesting to note further that the actual Q_{O_2} at the moment the addition was made was the same as that of the suspension in 0.3 per cent lactate where a further addition was ineffective. After nine hours, four hours after the respiration of the lactate suspension had become identical with that of the water control, the further addition of 0.2 per cent lactate to each gave the following Q_{O_2} values: water suspension, 19; lactate suspension, fourteen.

These observations strongly suggest that the falling off in rate of oxygen consumption with time is due less to accumulation of some retarding factor than to a temporary exhaustion of some required one. Certainly neither oxygen nor lactate are lacking, and no retarding substance accumulates as the ultimate product. Either some intermediate product is formed rapidly and but slowly further altered, so as to act as a temporary obstacle to the first reaction; or the oxidative mechanisms of the cell are somehow run down by an excessive load and are only gradually restored to the equilibrium state. The effects of sudden dilution are more in accord with the second possibility.

The effect of methylene blue combined with lactate is of interest in this connection. When, in comparable experiments, buffered solutions of lactate alone or with methylene blue are tipped into a water suspension, the maximum Q_{O_2} is consistently greater with the dye absent (Table III). In eight experiments with 0.1 per cent sodium lactate and 0.1 per cent methylene blue the maximum Q_{O_2} averaged 34; in four without the methylene blue, forty. In every run the depressant action of the dye was seen, decreases ranging from 10 to 30 per cent. At two to three hours the curves approached or even crossed for a short time, but at about six hours after the tipping the values were: with methylene blue, $Q_{O_2} = 0.9$, without, $Q_{O_2} = 2.5$.

Methylene blue has, to sum up, a marked early accelerating action on the respiration of *Sarcina* suspensions in water or in glucose solutions. After some hours the rate of oxygen consumption falls to that of control suspensions and is ultimately depressed. With lactate solutions, the depressant action of methylene blue is present at the start, and, in percentage, becomes more marked with the passage of time.

It may be pointed out that for equimolar amounts of glucose and sodium lactate added, the time course of extra oxygen consumption is very different. The glucose leads to a moderate prolonged rise,

the lactate to an intense brief one (Fig. 2). This would be in harmony with, though of course not necessitating, a slow split of the glucose molecule to lactic acid or a closely related intermediate followed by a rapid oxidation of this latter. Barron's view (1930) that methylene blue catalyzes primarily the initial splitting of glucose and not the later reactions would then fit the observed results fairly well. The main difficulty with such an interpretation lies in the failure to obtain an accumulation of lactic, pyruvic or other acid stronger than carbonic under anaerobic conditions, as shown in the preceding paper.

Finally, simultaneous addition of glucose and sodium lactate leads to the sharp maximum of lactate followed by the less intense enduring glucose effect. There does not appear to be summation of the two effects—the Q_{O_2} follows the higher single curve (lactate alone or glucose alone) at any time. No separate experiments were performed with optically active lactic acid, the racemate serving in these tests. Since, however, all added lactate can be burned and the Q_{O_2} curve after lactate addition gives no evidence of a discontinuity, it seems probable that both the *d* and *l* forms are easily utilized by this organism.

5. *Thioglycollic Acid.*—A few experiments, made in another connection, have shown that this substance in 0.2*M* concentration doubles the oxygen consumption.

6. *Sodium Cyanide.*—Cyanide, in concentrations up to *N*/100 or a little stronger does not inhibit the oxygen consumption of *Sarcina lutea*. This is true for the usual respiration of suspensions in water, saline, or phosphate buffer, as well as for the increases evoked by methylene blue, lactate, or glucose (Fig. 5). The presence of cyanide

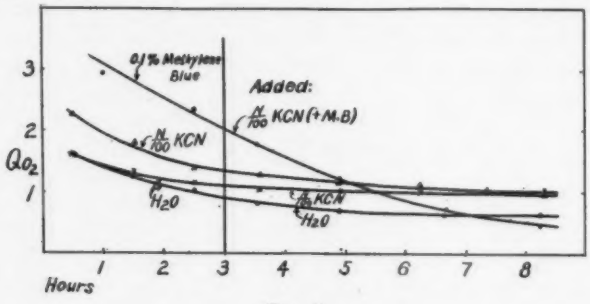

FIG. 5.

appears, in fact, to slightly increase the Q_{O_2} or at least to diminish the rate of fall with time. The average Q_{O_2} of five experiments in water was 1.1 at three hours; in *N*/100 NaCn it was 1.4. The possibility of HCN distilling from the side bulb to the main suspension

prematurely, and so masking a real effect, is excluded. In *N*/30 cyanide, respiration is depressed 1/2 to 2/3, and in *N*/10, 2/3 to 3/4. Rubinstein (1931) has confirmed the absence of cyanide inhibition with water suspensions.[1]

A much less complete absence of cyanide inhibition has been described by Emerson (1927) for the alga *Chlorella*, for in the presence of sugar 10^{-4} *M* cyanide gives 50 per cent inhibition. Lund (1918) has claimed a cyanide insensitivity for *Paramecium*, though this has not passed unchallenged (Hyman, 1919).[2] Pitts (unpublished) has found *Colpidium* to be but slightly sensitive to cyanide. Burnet (1927), studying growth and oxygen consumption of a number of bacterial types under the influence of cyanide, found them divisible into two groups. Most showed a usual degree of sensitivity, but some, as streptococci, were insensitive to cyanide. These groups were also different in their behavior with hydrogen peroxide. Even for vertebrate tissues a complete inhibition of respiration by cyanide is not the rule. *N*/100 does not inhibit the respiration of frog nerve by more than 80 per cent (Gerard, 1930), and Dixon and Elliot (1929) obtained similar results with a variety of mammalian tissues, though Alt (1930) and Warburg (1930) sharply criticize their work. The objections raised certainly do not apply to the present results. *Sarcina lutea* apparently carried on a fairly typical aerobic metabolism, using oxygen freely to burn the usual organic molecules. The almost complete insensitivity of its respiratory catalytic system to cyanide would seem to preclude a too broad generalization as to the ferroactive nature of oxidative enzymes.[3] The action of carbon monoxide is likewise atypical.

7. *Carbon Monoxide.*—Carbon monoxide containing 5 per cent

[1] Working with descendants of the original strain, over a year later, Barron obtained definite inhibition of respiration by cyanide. I repeated my original experiments, using the identical techniques previously employed, and also found inhibition was now present. In water suspensions *N*/100 cyanide gave depressions of 50 per cent or more, in glucose even greater ones. The depressions were, however, of short duration and respiration returned to normal in an hour and sometimes became excessive later. Presumably the organisms had altered in the interim due, perhaps, to unknown differences in the culture conditions.

[2] This has now been confirmed. Gerard and Hyman, *Am. Jour. Physiol.*, in press.

[3] It may be mentioned here that Dr. E. S. G. Barron and I have demonstrated by means of the spectroscope that cytochrome is present in these cells. Further, it is changed easily between the oxidized and reduced states by bubbling in oxygen or nitrogen. In the presence of *N*/100 KCN, however, the pigment remains reduced even in a stream of oxygen. This appears as evidence that cytochrome is not an essential link in the chain of respiratory substances of this cell, since respiration proceeds as normally after its oxidation is blocked. We are further investigating the oxidizing mechanisms of *Sarcina*.

oxygen was used in all experiments and the thermostat covered to exclude daylight. A 200-watt tungsten bulb, immersed in the white-walled thermostat, within 2 to 10 inches of the manometer chambers, was turned on at intervals of 20 minutes to 1 hour.

Water suspensions of *Sarcina* respired alike in air and in the monoxide mixture. Light had no obvious effect on either. When glucose (1.0 per cent) or lactate (1.0 per cent) was added to such water suspensions, an inhibiting action of the CO appeared. In the case of glucose this was slight, the increased oxygen consumption in CO being over three-fourths that in air. With lactate, the inhibition was marked, the increase in monoxide being less than one-fifth that in air, but inadequate oxygen may have contributed to the effect. In both cases, glucose or lactate added, when the carbon monoxide

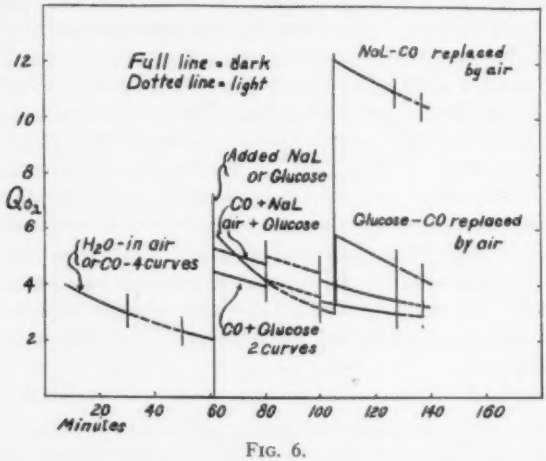

FIG. 6.

mixture was later replaced by air, the full rate of oxygen consumption promptly appeared. The presence of light, of the intensity used, had no reversing action on the inhibited lactate oxidation. There was a suggestion of such an effect in increasing the respiration of a suspension in glucose in the presence of CO, but an entirely similar augmentation appeared also in air (Fig. 6). Rubenstein (1931) has found that light increases the respiration of *Sarcina lutea* at this temperature.

DISCUSSION

These results raise several interesting questions regarding the chemical dynamics involved. Q_{O_2} is a measure of reaction velocity, yet this is independent of the concentration of the primary reactants

over a wide range. Thus 0.05 and 1.1 per cent lactate give the same maximal rates, but both rates decline following the maximum. For the lower concentrations, at least, the rate must fall with decreasing amount of substrate. Probably at sufficiently low initial concentrations, the maximum reached would vary with concentration, until an asymptotic value had been reached, beyond which concentration has no effect. The critical concentration of lactate cannot be over 0.05 per cent, but may be very close to this. The decline in rate after the maximum, when considerably larger amounts of lactate are present, cannot, as in the previous case, be due to diminution in concentration of the lactate, for this must remain for long periods above the critical value. Evidence has also been presented to show that accumulation of end products is not the important factor leading to slowing, though temporary piling up of intermediates might play a rôle. Since, in the simplified reaction:

$$\text{Substrate} + O_2(+ \text{ Respiratory Catalyst}) \rightarrow CO_2 + H_2O + \text{End Products},$$

the velocity appears to be independent of substrate, oxygen, or end product concentration, its fall might be attributed to interference with the respiration-catalyzing system. This might mean destruction or out-diffusion (suggested by the fall in Q_{O_2} on dilution) from the cell of one of the key substances in the respiratory chain more rapidly than it is replaced.

The situation with respect to glucose is analogous though quantitatively different, and the ultimate depressant action of methylene blue can also be laid at the door of an injured catalytic system.

Meyerhof (1912) has shown that the oxygen consumption of acetone-yeast is doubled by the addition of methylene blue, whereas that of the intact cells is depressed by it; and similar results were obtained with staphylococci (1917). Barron (1930) likewise has found little or no stimulating action of methylene blue on normal tissues, though it does increase the respiration of cancer and other aerobically glycolysing material. Gerard (1930) has found that medullated nerve, on the contrary, though lacking aerobic glycolysis, is stimulated by methylene blue to a marked respiratory increase, and Chang (unpublished) has confirmed this for non-medullated nerve and with cresyl blue. *Sarcina* in this respect resembles nerve. It may be noted in passing that methylene blue largely or entirely reverses the cyanide inhibition of respiration in the case of staphylococci (Meyerhof) and nerve (Gerard, Chang). There is no evidence that the yellow pigment of *Sarcina* acts in a similar way, though such activity might contribute to the cyanide insensitivity of this organism.

Summary

The Q_{O_2} of washed *Sarcina lutea* in water suspension at 22° C. in the relatively steady state averages 2.5. There is no untoward effect of crowding in heavy suspensions. The presence of NaCl does not modify the Q_{O_2} until $M/5$ concentrations are reached, when a slight depression may result. The Q_{O_2} is also unaffected by pH, at least between 7 and 8, or by the presence of phosphate buffer mixtures.

Glucose addition causes a marked increase in the Q_{O_2}, the maximum being largely independent of glucose concentration. The extra oxygen used under the influence of glucose may be more than that required to fully oxidize it which, with other evidence, suggests a "specific dynamic action."

Sodium lactate may increase the Q_{O_2} in water suspension over twenty-fold. Its addition is always followed by a great rise of the respiratory rate, the maximum reached being independent of lactate concentration, at least between 0.05 and 2.0 per cent. The respiration falls rapidly back to normal after addition of small amounts, and the extra oxygen consumed accounts for full oxidation of the added lactate, both *d* and *l* forms. With larger concentrations of lactate, the increased respiration also falls after the initial maximum, but more slowly. This fall is not primarily due to removal of lactate nor accumulation of end products.

Methylene blue added to a suspension in water doubles respiration at first, later depresses it. Added to one in glucose solution the same sequence appears. The Q_{O_2} in glucose is increased only 50 per cent by the dye, but this is an absolute increase over twice that in water; so that glucose and methylene blue added together to a water suspension cause a greater increase in respiration than the sum of their separate effects. The dye added to lactate solutions seems to be depressant from the start.

Thioglycollic acid doubles the respiration of a buffered water suspension.

Sodium cyanide causes no inhibition of respiration up to concentrations of $M/100$ or somewhat stronger. This is true for the low respiration of suspensions in water, saline, or phosphate buffer and for the increases evoked by methylene blue, lactate and glucose. Extremely strong cyanide concentrations do depress, but even $M/10$ NaCN does not abolish more than 2/3 to 3/4 of the total respiration.

Carbon monoxide containing 5 per cent oxygen has no effect on the respiration of a water suspension, but somewhat inhibits the

increase in glucose and largely that in lactate. Light has little if any effect on the inhibition.

The metabolism of *Sarcina lutea* is compared with that of other cell types.

My thanks are due Miss Ruth Bilger for valuable technical assistance.

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THE OXYGEN TENSION-OXYGEN CONSUMPTION CURVE OF UNFERTILIZED ARBACIA EGGS

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Gerard (1931) has developed equations relating the external oxygen pressure to the oxygen consumption, cell diameter and diffusion constants for individual cells. This work was undertaken to obtain experimental data under favorable conditions for comparison with the theoretical derivation. For this the sea urchin egg is beautifully adapted, as it is homogeneously spherical, regular, and its resting rate of oxygen consumption can be suddenly increased by fertilization. Its effective diameter can also be varied by allowing a greater or lesser amount of the egg jelly to remain about each cell. It is our intention to investigate the effect of varying these several factors, but for the present the results with simple, clean, unfertilized eggs are to be presented.

METHODS

Oxygen consumption was determined by the usual Warburg technique, using small conical vessels of about three cc. capacity, containing an inset for alkali. The experiments were conducted at 24.7° C. and the shaking of the manometers was demonstrated to be adequate for diffusion equilibrium even at the low oxygen pressures used. Gas mixtures were prepared by mixing nitrogen and air over water and, for the smaller concentrations, by direct mixing in the manometer as described by Gerard and Falk (1931). When the mixing was done over water, the composition was checked by analysis in the Haldane apparatus. Passing the gas through the manometers with gentle shaking for fifteen minutes sufficed to bring the fluid into complete equilibrium with it.

The *Arbacia* eggs were obtained by picking out the gonads of about twenty animals, allowing them to shed into sea water, and straining through cheese cloth. The eggs were allowed to settle and the supernatant water decanted, giving a heavy suspension. They were not further washed, but controls showed no difference in respiration between washed and unwashed suspensions, and fertilization tests

[1] It is a pleasure to acknowledge the guidance, help and suggestions of Professors R. S. Lillie and R. W. Gerard, under whose supervision this work was done. To Dr. Walter S. Root my thanks are due for many of the gas analyses.

yielded over 90 per cent cleavage. The concentration of eggs in the suspension was determined by means of a hemocytometer, and half a cc. was used for each manometer. The egg diameters, as determined by an ocular micrometer, averaged 77 micra, variation 72 to 80.

Significant readings were made for three and a half to four hours and the viability of the eggs again checked by fertilization. In each experiment parallel runs were made of the respiration in air or oxygen and at the lower oxygen pressures, so that the latter values could be expressed as a direct percentage of the former.

TABLE I

Oxygen Tension mm. of Hg	Oxygen Consumption per cent in air	No. of Experiments
2.0	43.0	13
6.1	54	7
9.9	69	2
20.5	92	3
42.0	92	5
86.0	101	3
160 (air)	100 ($Q_{O_2} = 33.6$)	24
760	100	3

RESULTS

The average Q_{O_2}, expressed as cubic millimeters of oxygen consumed per million eggs per hour, was 33.6 in air (extremes 17 to 51; 24 experiments), the lower values being obtained toward the end of the breeding season. At oxygen tensions of 70–80 mm. or higher, up to pure oxygen, the consumption was the same as in air. Below 70–80 mm. the consumption fell off with pressure, as indicated in Table I. Beginning at the oxygen tension of 20 mm., the oxygen consumption fell very rapidly with a decrease in oxygen tension. Surprisingly, the same type of curve, with the turning point coming at the same oxygen tension has been obtained by Amberson (1928), on *fertilized* eggs by a different method.

The oxygen consumption was found to proceed at a constant rate up to about four and one-half hours, at which time there was a sudden increase in oxygen consumption, both in air and in lower concentrations of oxygen. This seems to be related to a fairly abrupt cytolysis of large numbers of the cells. A few experiments were carried out with fertilized sea urchin eggs to determine the possible existence of a rhythm of oxygen consumption analogous to the rhythm of production of CO_2 reported by Lyon (1904). The experiments showed the

fertilized eggs to have a respiration five times that of the unfertilized ones, confirming Warburg (1908) and Loeb and Wasteneys (1910); but, within the limits of accuracy of the method, the rate of respiration was constant through the first, second and third cleavage periods.

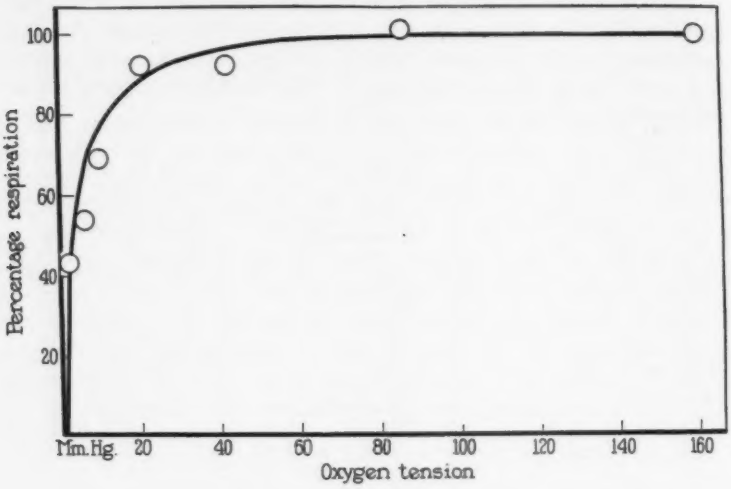

FIG. 1.

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OXYGEN DIFFUSION INTO CELLS

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Krogh (1919) determined the coefficients of diffusion of oxygen through surviving frog muscle and through fascia by using these as tissue membranes and measuring the gas diffusing through under given conditions. He applied the measured values in a formula relating capillary size and number to the oxygen needs of muscle *in vivo*.

These constants have been utilized by subsequent workers for substitution in other equations applying to the equilibrium state. Warburg (1923) developed an equation relating the necessary oxygen pressure to the metabolism and thickness of a slice of isolated tissue. Fenn (1927) and Gerard (1927) independently presented analogous equations for a cylinder of tissue, and A. V. Hill (1928) has extensively surveyed the general question of diffusion in tissues and developed several formulæ for the attaining of equilibrium as well as for the equilibrium state. Applying these equations to the specific question of diffusion of phosphate ion (Stella, 1928) and lactate ion (Eggleton, Eggleton and Hill, 1928), the values for the diffusion constants obtained suggested that these ions moved mainly in the intercellular fluid and with much greater difficulty across cell boundaries.

Should a similar situation obtain for oxygen, the calculations based on Krogh's constant might be considerably in error, and in any case, since this value has been so widely used, any further checks on its magnitude should be of value.

The simplest situation for studying oxygen penetration into living cells is offered, of course, by the unicellular organisms, which present, mainly, the problem of the sphere. It was the original intent of this work to evaluate the diffusion constant for cells as the only unknown in a simple equation, but it soon became apparent that a more searching examination of the assumptions made in such a simplified derivation was necessary. The elementary equation for diffusion into a sphere is easily derived as follows:

At equilibrium, the amount of oxygen diffusing into a mass of tissue must equal the amount consumed by it in the same time. For unit time, the inward diffusion depends on the surface area ($4\pi r^2$ for the sphere); the diffusion coefficient, D; and the gradient

of oxygen concentration along a radius, dC/dr, C being the oxygen concentration at radius r. The consumption of oxygen by a sphere of indeterminate radius, r, is the product of the measured consumption per unit volume $\left(= \frac{\text{mass}}{\text{sp. gr.}} = \text{mass} \times 0.95 \text{ for most protoplasm}\right)$, A, times the volume, $\frac{4}{3}\pi r^3$. At equilibrium, then

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 A$$

or

$$\frac{dC}{dr} = \frac{Ar}{3D}; \tag{1}$$

which gives on integration

$$C = \frac{Ar^2}{6D} + K.$$

At $r = 0$, $C = K$ and K therefore represents the oxygen concentration at the center of the sphere.

In the case of most tissues so far studied, the oxygen consumption, A, has been found to be independent of the oxygen pressure over a wide range. The assumption has been made in this derivation that consumption in any one region is independent of oxygen concentration, provided this is greater than zero. Then for the critical condition where the oxygen concentration just reaches zero at the center of the sphere, $K = 0$, and the oxygen concentration at any level in the sphere is

$$C = \frac{Ar^2}{6D};$$

and at the surface, $r = r_0$, the critical concentration of oxygen needed to just insure a supply at the center is

$$C_0 = \frac{Ar_0^2}{6D} \tag{2}$$

and

$$D = \frac{Ar_0^2}{6C_0}. \tag{3}$$

A may be fairly simply determined with one of the available methods of measuring oxygen consumption, and C_0 is that concentration of oxygen in the medium which just gives full values of oxygen consumption. With lower concentrations the observed oxygen consumption begins to fall off. If the radius of the cell is known, a simple

means is available for obtaining the oxygen diffusion coefficient, or for bringing out the invalidity of assumptions as to the conditions of respiration.

It may be stated at once that the results of applying this simple equation to a variety of cells, as compared with results experimentally obtained, show such great variation as to indicate that other factors than the simple diffusion constants must be involved. It is necessary, then, to examine in some detail the assumptions made in the above derivation. Probably the most important is that just expressed, namely that the oxygen consumption of any region is independent of the concentration of oxygen at that region. This will be examined later. Other assumptions of little probable importance in the case of tissues may become extremely significant in the case of the individual cell. These are, first, that the oxygen consumption per individual region is a constant throughout the cell, that is, that the total consumption divided by the total volume gives the true consumption of each region. This is certainly not necessarily true, as the rate of respiration of a nuclear region or a cortical region might clearly be very much greater than that in other regions. It is also implicitly assumed that the diffusion constant, whether equal to that of Krogh or not, is at least constant throughout the cell. This also is not at all certain; in fact, in view of the previous discussion, it is entirely possible that the constant would vary widely across the membrane and in the cell interior.[1]

The mathematical development which follows is designed first to include all possible cases in which diffusion or consumption in two concentric regions of the cell are not assumed to be equal; and, following that, the more difficult case of the dependence of consumption on oxygen concentration will be considered. In this treatment I have called freely upon the expert assistance, generously given, of Dr. Walter Bartky of the Department of Astronomy at this University. The final section of the paper will apply the equations derived to the data available in the literature as well as to our own measurements, in an attempt to evaluate the conditions actually existing as regards respiration. Obviously, the derivations apply equally to other diffusable substances formed in and leaving or entering and utilized by the cell.

[1] The further complication, in many cells, of protoplasmic streaming cannot be handled rigorously. It is probably not significant in bacteria, yeast, or even *Arbacia* eggs; and when it is present, the stirring action would act as a decrease in radius in lowering the diffusion limit.

1. *Oxygen Consumption not Alike in All Portions of A Sphere*

a. Limited to a central core (nuclear respiration).—Let Q = the total oxygen consumed by the cell; C = the oxygen concentration at radius, r; r_0 = radius at cell surface; r' = radius of respiring region.

The oxygen diffusing into a sphere centered at the cell center and of any radius, r, equals $4\pi r^2 D \frac{dC}{dr}$. When $r \geqq r'$, all oxygen consumed by the cell must pass through to the region of consumption, so:

$$4\pi r^2 D \frac{dC}{dr} = Q.$$

The consumption per unit volume in the non-respiring portion of the cell is zero; of the respiring portion: $\frac{Q}{\frac{4}{3}\pi r'^3}$.

When $r \leqq r'$, then, the oxygen passing the shell must equal the volume within this shell times the consumption per unit volume, or

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 \frac{Q}{\frac{4}{3}\pi r'^3}.$$

Then

$$\frac{dC}{dr} = \frac{Q}{4\pi r^2 D}, \qquad \text{when} \quad r \geqq r', \tag{4a}$$

$$\frac{dC}{dr} = \frac{rQ}{r_0^3\, 4\pi D}, \qquad \text{when} \quad r \leqq r'. \tag{4b}$$

Integrating:

$$C = -\frac{Q}{4\pi D r} + K_1, \qquad r \geqq r', \tag{5a}$$

$$C = \frac{Q r^2}{8\pi D r_0^3} + K_2, \qquad r \leqq r'. \tag{5b}$$

For the critical condition, $C = 0$ when $r = 0$ and $C > 0$ for $r > 0$, then $K_2 = 0$; and since C is continuous at $r = r'$,

$$-\frac{Q}{4\pi D r'} + K_1 = \frac{Q r'^2}{8\pi D r'^3}$$

or

$$K_1 = \frac{3Q}{8\pi D r'}.$$

So that, from (5*a*),

$$C = \frac{Q}{4\pi D}\left(\frac{3}{2r'} - \frac{1}{r}\right), \qquad r \geqq r'.$$

At the cell surface $r = r_0 \geqq r'$,

$$C_e = \frac{Q}{4\pi D}\left(\frac{3}{2r'} - \frac{1}{r_0}\right). \tag{6}$$

(C_e = oxygen concentration needed at the surface, radius = r_0, to just insure full penetration, in this case when all the oxygen is consumed within a sphere of radius r'.)

For the special case when $r' = r_0$, or oxygen consumption is uniform throughout the cell, this reduces to

$$C_0 = \frac{Q}{8\pi D r_0}$$

or, since A = consumption per unit volume,

$$A = \frac{Q}{\frac{4}{3}\pi r_0^3},$$

and therefore

$$C_0 = \frac{A r_0^2}{6D},$$

the original equation.

In general,

$$\frac{C_e}{C_0} = \frac{3\,r_0}{r'} - 2. \tag{7}$$

It is readily seen from equation (7) that as r' decreases relative to r_0, the critical oxygen pressure needed, C_e, increases rapidly relative to C_0. When $r' = \frac{1}{2}r_0$, $C_e = 4C_0$; when $r' = \frac{1}{4}r_0$, $C_e = 10C_0$.

b. Oxygen consumption limited to an outer shell (cortical region).—By an entirely analogous derivation it can be shown for this case that

$$\frac{C_e}{C_0} = \frac{(r_0 + 2r')(r_0 - r')}{r_0^2 + r_0 r' + r'^2}. \tag{8}$$

From this equation it appears that as the respiring shell of thickness $(r_0 - r')$ thins, $\frac{C_e}{C_0}$ decreases towards zero. When

$$r' = 0, \frac{C_e}{C_0} = 1;\quad r' = \tfrac{1}{2}r_0, \frac{C_e}{C_0} = 0.57;\quad r' = \tfrac{3}{4}r_0, \frac{C_e}{C_0} = 0.27;$$

$$r' = 0.9r_0, \qquad \frac{C_e}{C_0} = 0.10.$$

c. Oxygen consumption present throughout cell, but more or less intense in an outer shell.—This will be considered in the general case below. See Equations 17 and 18.

2. *Diffusion Constant Different in Central and Cortical Parts of Cell*

If the diffusion constant is smaller at one region than another—as that of a relatively impermeable membrane is small compared to that of a permeable cell interior—the following derivation becomes necessary.

Let the diffusion coefficient be D' for the cell interior from $r = 0$ to $r = r'$; and D for a superficial shell, $r = r'$ to $r = r_0$. Then, by a familiar derivation,

$$\text{When} \quad r \leqq r', \qquad 4\pi r^2 D' \frac{dC}{dr} = \frac{4}{3}\pi r^3 A; \qquad \frac{dC}{dr} = \frac{Ar}{3D'}$$

$$\text{When} \quad r \geqq r', \qquad 4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi r^3 A; \qquad \frac{dC}{dr} = \frac{Ar}{3D}.$$

Integrating between limits, and assuming $C = 0$ for $r = 0$, and $C > 0$ for $r > 0$,

$$C' = \frac{Ar'^2}{6D'},$$

$$C_0 - C' = \frac{A(r_0^2 - r'^2)}{6D},$$

where C' is the concentration at r'. Combining,

$$C_0 = \frac{A}{6}\left(\frac{r_0^2 - r'^2}{D} + \frac{r'^2}{D'}\right), \tag{9}$$

which reduces to the simple equation (2) when $D = D'$ or $r' = r_0$.

It is obvious from (8) that if the cell membrane is thin compared to the cell radius, that is, r' is nearly as large as r_0, its diffusion constant, D, must be very small in relation to that of the interior, D', to affect C_0. For example, to double C_0 when $r' = 0.9r_0$, D must be $0.16D'$; and when r' is $0.99r_0$, $D = 0.02D'$. To increase C_0 ten times: for $r' = 0.9r_0$, $D = 0.012D'$; for $r' = 0.99r_0$, $D = 0.002D'$. Since it appears from the work of the Eggletons and Hill (1928) that the diffusion coefficient for ions across the cell surface may be one hundredth as large as in the cell or intercellular fluid, this factor obviously might be of importance for oxygen diffusion. But unionized molecules appear to penetrate membranes much more easily than do ions, so that the analogy may not hold.

In all the above derivations the critical oxygen pressure at the cell surface has been calculated. That is, assuming that the oxygen consumption of the whole cell remains at its maximum value, what is the necessary external pressure as the various conditions within the

cell are changed? It is next desirable to investigate the oxygen consumption-external oxygen pressure curve below this critical value and to examine its shape for the simplest situation.

Let r_1 represent the depth of oxygen penetration, *i.e.*, $C = 0$ for values of r such that $r_1 \geqq r \geqq 0$, and $C > 0$ for $r > r_1$. And let C_s represent the oxygen concentration at the surface, $r = r_0$.

Then, as the respiring volume $= \frac{4}{3}\pi(r^3 - r_1^3)$,

$$4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi A(r^3 - r_1^2),$$

$$\frac{dC}{dr} = \frac{A(r^3 - r_1^3)}{3Dr^2},$$

$$C_s = \frac{A}{3D}\int_{r_1}^{r_0} \frac{r^3 - r_1^3}{r^2},$$

$$= \frac{A}{3D}\left(\frac{r_0^2}{2} + \frac{r_1^3}{r_0} - \frac{3}{2}r_1^2\right). \tag{10}$$

Similarly, the oxygen consumed is:

$$Q = 4\pi \int_{r_1}^{r_0} Ar^2 dr,$$

$$Q = \frac{4\pi A}{3}(r_0^3 - r_1^3). \tag{11}$$

Take units such that $r_0 = 1$, and that $C_s = C_0 = 1$ for $r_1 = 0$, and also that $Q = 1 = Q_0$ for $r_1 = 0$. Note that with this selection of units, $\frac{A}{D} = 6$, $A = \frac{3}{4\pi}$, $D = \frac{1}{8\pi}$. The relation between Q and C for the general case is then expressed in terms of r as a parameter: From (10)

$$C_s = 1 - r_1^2(3 - 2r_1),$$

and from (11)

$$Q = 1 - r_1^3,$$

which give the following values (Table I). The corresponding curve is given in Fig. 1.

Still more generally, assuming only that A is constant as regards C, the following derivation includes all cases involving a single concentric zoning of the cell as regards respiration and diffusion.

Let r' represent the surface separating two cell zones.

Let D' and A' represent the diffusion coefficient and unit oxygen consumption for the nuclear region, $r < r'$; and similarly D and A represent these values for the cortical region, $r > r'$.

TABLE I

$\frac{C_e}{C_0}$	$\frac{r_1}{r_0}$	$\frac{Q}{Q_0}$
1.00	0.0	1.00
0.90	0.2	0.99
0.65	0.4	0.94
0.50	0.5	0.87
0.35	0.6	0.78
0.22	0.7	0.66
0.10	0.8	0.49
0.04	0.9	0.27
0.00	1.0	0.00

Let r_1 represent, as previously, the depth of oxygen penetration. In the following it is assumed that $r_1 \leqq r'$, for when $r_1 > r'$ the situation reduces to the simple cases previously considered, inasmuch as no oxygen reaches the inner sphere where conditions are different.

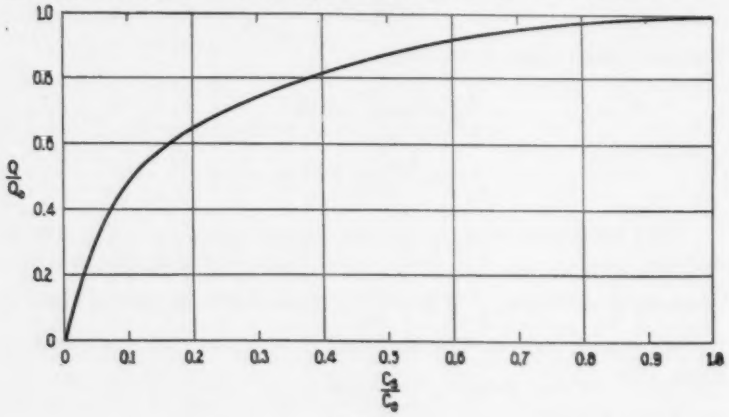


FIG. 1.

Let C' represent the oxygen concentration at $r = r'$; C_e the oxygen concentration at the cell surface, $r = r_0$; and C_c the critical surface concentration to just insure complete penetration. For the simple case, $C_e = C_0$.

Then the derivation, along the previous lines, is:

$$\text{For } r < r', \qquad 4\pi r^2 D' \frac{dC}{dr} = \frac{4}{3}\pi A'(r^3 - r_1^3); \tag{12a}$$

$$r > r', \qquad 4\pi r^2 D \frac{dC}{dr} = \frac{4}{3}\pi(r'^3 - r_1^3)A' + \frac{4}{3}\pi A(r^3 - r'^3). \tag{12b}$$

Simplifying:

$$\text{For } r < r', \qquad \frac{dC}{dr} = \frac{A'}{3D'} \frac{(r^3 - r_1^3)}{r^2},$$

and integrating from r_1 to r':

$$C' = \frac{A'}{3D'} \left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3r_1^2}{2} \right). \tag{13}$$

Similarly:

$$\text{For } r > r', \qquad \frac{dC}{dr} = \frac{A'(r'^3 - r_1^3)}{3Dr^2} + \frac{A(r^3 - r'^3)}{3Dr^2}$$

and integrating from r' to r_0:

$$C_s - C' = \frac{A'(r'^3 - r_1^3)}{3D} \left(\frac{1}{r'} - \frac{1}{r_0} \right) + \frac{A}{3D} \left(\frac{r_0^2}{2} - \frac{r'^3}{r_0} - \frac{3}{2} r'^2 \right). \tag{14}$$

From (13) and (14)

$$C_s = \frac{A'(r'^3 - r_1^3)}{3D} \left(\frac{1}{r'} - \frac{1}{r_0} \right) + \frac{A}{3D} \left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2} r'^2 \right) + \frac{A'}{3D'} \left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3}{2} r_1^2 \right). \tag{15}$$

Equation (15) may be explored as follows:

For $r_1 = 0$, that is, at the critical external oxygen pressure,

$$C_c = \frac{A'r'^3}{3D} \left(\frac{1}{r'} - \frac{1}{r_0} \right) + \frac{A}{3D} \left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2} r'^2 \right) + \frac{A'r'^2}{6D'}. \tag{16}$$

For $r_1 = r' = 0$

$$C_c = \frac{Ar_0^2}{6D}.$$

For $r_1 = 0$, $r' = r_0$

$$C_0 = \frac{A'r_0^2}{6D'}.$$

For $r_1 = r'$

$$C_s = \frac{A}{6D} \left(r_0^2 + \frac{2r'^2}{r_0} - 3r'^2 \right). \quad \text{(See Equation 10.)}$$

For $r_1 = r' = r_0$

$$C_s = 0.$$

For $D = D'$

$$C_s = \frac{A'}{3D} \left[\frac{3}{2} (r'^2 - r_1^2) - \frac{r'^3 - r_1^3}{r_0} \right] + \frac{A}{3D} \left(\frac{r_0^2}{2} + \frac{r'^3}{r_0} - \frac{3}{2} r'^2 \right)$$

$$= \frac{(A - A')r'^2}{6D} \left(\frac{2r'}{r_0} - 3 \right) + \frac{Ar_0^2}{6D} + \frac{A'r_1^2}{6D} \left(\frac{2r_1}{r_0} - 3 \right). \tag{17}$$

If $A = A'$, or $r_1 = r'$,

For $r_1 = 0$, $\quad C_s = \frac{A}{6D}\left(r_0^2 + \frac{2r_1^3}{r_0} - 3r_1^2\right)$. (See Equation 10.)

$$C_c = \left(\frac{A - A'}{6D}\right) r'^2 \left(\frac{2r'}{r_0} - 3\right) + \frac{Ar_0^2}{6D}. \tag{18}$$

If $A = A'$,

$$C_0 = \frac{Ar_0^2}{6D}.$$

For $A = A'$

$$C_s = \frac{A}{3D}\left[\frac{r_0^2 - r'^2}{2} - r_1^3\left(\frac{1}{r'} - \frac{1}{r_0}\right)\right] + \frac{A}{3D'}\left(\frac{r'^2}{2} + \frac{r_1^3}{r'} - \frac{3}{2}r_1^2\right)$$

$$= \frac{A}{6}\left(\frac{1}{D'} - \frac{1}{D}\right)\left(r'^2 + \frac{2r_1^3}{r'}\right) + \frac{A}{6}\left(\frac{r_0^2 - \frac{2r_1^3}{r_0}}{D} - \frac{3r_1^2}{D'}\right). \tag{19}$$

If $D = D'$ or $r_1 = r'$,

For $r_1 = 0$, $\quad C_s = \frac{A}{6D}\left(r_0^2 - \frac{2r_1^3}{r_0} - 3r_1^2\right)$ (See Equation 10.)

$$C_c = \frac{A}{3D}\left(\frac{r_0^2 - r'^2}{2}\right) + \frac{Ar'^2}{6D'}$$

$$= \frac{A}{6}\left(\frac{r_0^2 - r'^2}{D} + \frac{r'^2}{D'}\right). \qquad \text{(See Equation 9.)}$$

If $D = D'$,

$$C_0 = \frac{Ar_0^2}{6D}.$$

The expression for oxygen consumed per cell is:

$$Q = 4\pi A' \int_{r_1}^{r'} r^2 dr + 4\pi A \int_{r'}^{r_0} r^2 dr$$

$$= \frac{4}{3}\pi[A'(r'^3 - r_1^3) + A(r_0^3 - r'^3)]. \tag{20}$$

Table II and Fig. 2 give the $Q - C_s$ curves obtained for various relationships of $\frac{r'}{r_0}, \frac{D}{D'}, \frac{A}{A'}$.

Consider finally the general case where $A \alpha F(C)$ and is not always a constant, though approximately so for high values of C_0. The amount of oxygen crossing a spherical surface of radius r is $4\pi r^2 D \frac{dC}{dr}$;

therefore that consumed in a shell of internal radius = r, external radius = $r + dr$, is

$$4\pi \left(r^2 D \frac{dC}{dr} \right) \Big|_{r+dr} - 4\pi \left(r^2 D \frac{dC}{dr} \right) \Big|_{r}.$$

This is equal to the consumption per unit volume times the volume: $A 4\pi r^2 dr$.

A is some function of $C = DF(C)$, where $F(C)$ does not decrease with increase in C, *i.e.*, a monotonically increasing function of C. Then

$$\left(r^2 D \frac{dC}{dr} \right) \Big|_{r+dr} - \left(r^2 D \frac{dC}{dr} \right) \Big|_{r} = DF(C) r^2 dr$$

or

$$\frac{d\left(r^2 D \frac{dC}{dr} \right)}{dr} = r^2 DF(C);$$

and, assuming D independent of C and r,

$$\frac{d\left(r^2 \frac{dC}{dr} \right)}{dr} = r^2 F(C). \tag{21}$$

The total oxygen consumption of a cell is

$$Q = \int_0^{r_0} A 4\pi r^2 dr$$

$$= 4\pi D \int_0^{r_0} F(C) r^2 dr. \tag{22}$$

Q as a function of C_0 is an experimentally observable relationship, from which it is desired to obtain $F(C)$. Consider the most general case, that is, either $C = 0$ for $r = r_1$, where $r_1 \geqq 0$, or $C > 0$ for all values of r. For this latter, always take $r_1 = 0$ in what follows. Integrating (21) between r_1 and r,

$$r^2 \frac{dC}{dr} = \int_{r_1}^{r} r^2 F(C) dr,$$

$$\frac{dC}{dr} = \frac{1}{r^2} \int_{r_1}^{r} r^2 F(C) dr;$$

and integrating between r and r_0,

$$C_0 - C = \int_r^{r_0} \frac{1}{r^2} \left(\int_{r_1}^{r} r^2 F(C) dr \right) dr,$$

or

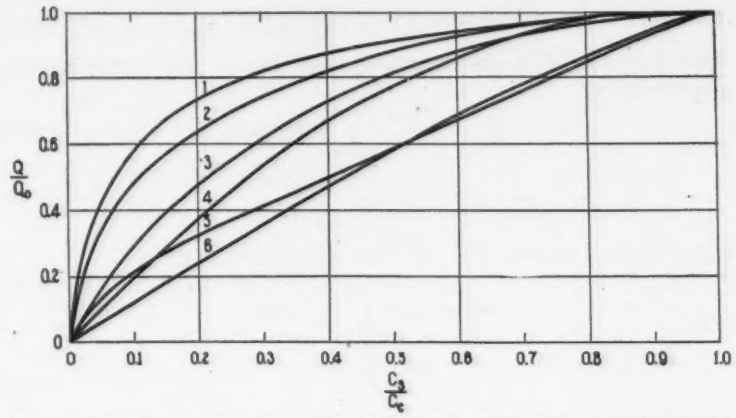
$$C = C_0 - \int_r^{r_0} \frac{1}{r^2} \left(\int_{r_1}^{r} r^2 F(C) dr \right) dr. \tag{23}$$

TABLE II

For $A = A'$						$A = 5A'$					
$\frac{Q}{Q_0}$	C_s/C_c										
	$r' = 0.9\ r_0$ $D = 0.01\ D'$	$r' = 0.98\ r_0$ $D = 0.1\ D'$	$r' = 0.67\ r_0$ $D = 0.01\ D'$	$D = D'$	$r' = 0.98\ r_0$ $D = 0.01\ D'$	$r' = 0.67\ r_0$ $D = D'$		$r' = 0.98\ r_0$ $D = 0.1\ D'$		$r' = 0.9\ r_0$ $D = 0.01\ D'$	
						$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(1.00)	(19.8)	(1.37)	(56.1)	(1.00)	(5.03)	(3.83)	(2.04)	(1.24)	(1.46)	(2.08)	(3.10)
0.99	0.97	0.945	0.98	0.90	0.96	1.00	0.95	1.00	0.92	1.00	0.99
0.94	0.91	0.72	0.88	0.65	0.86	0.99	0.83	0.95	0.70	0.97	0.95
0.875	0.85	0.60	0.77	0.50	0.80	0.98	0.75	0.90	0.62	0.94	0.90
0.79	0.73	0.47	0.61	0.35	0.69	0.945	0.68	0.83	0.50	0.90	0.83
0.66	0.58	0.33	0.38	0.22	0.56	0.80	0.27	0.72	0.37	0.84	0.73
0.49	0.39	0.21	0.18	0.10	0.40	0.64	0.13	0.59	0.25	0.76	0.61
0.27	0.15	0.09	0.05	0.03	0.21	0.35	0.035	0.41	0.16	0.65	0.45
0.14	0.05	0.04	0.02	0.01	0.11	0.19	0.01	0.31	0.10	0.36	0.15
	Fig. 2, Curve 5	Curve 3		Curve 2		Fig. 2, Curve 1					

$A = 20\ A'$				$A = 1/5\ A'$					
$r' = 0.9\ r_0$ $D = 0.1\ D'$		$r' = 0.98\ r_0$ $D = 0.1\ D'$		$r' = 0.5\ r_0$ $D = D'$		$r' = 0.9\ r_0$ $D = 0.1\ D'$		$r' = 0.98\ r_0$ $D = 0.01\ D'$	
$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$	$\frac{Q}{Q_0}$	$\frac{C_s}{C_c}$
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(6.13)	(8.00)	(2.12)	(1.76)	(0.3)	(0.61)	(0.79)	(2.98)	(0.96)	(4.85)
1.00	1.00	1.00	0.95	0.95	0.82	0.99	0.82	0.99	0.98
0.99	0.94	0.97	0.80	0.79	0.40	0.92	0.68	0.93	0.88
0.98	0.91	0.94	0.70	0.58	0.17	0.84	0.58	0.86	0.80
0.97	0.87	0.90	0.60			0.73	0.45	0.77	0.69
0.95	0.82	0.84	0.50			0.56	0.33	0.64	0.55
0.92	0.76	0.76	0.40			0.35	0.19	0.46	0.39
		0.65	0.31					0.23	0.19
		0.59	0.27					0.10	0.08
						Curve 4		Curve 6	

C_s is the largest value of C and hence $F(C_s)$ is the largest value $F(C)$ can have. Replacing $F(C)$ by this constant maximum value and also, in the case $r \neq 0$, replacing r by 0 (this gives a larger range



Curve	$\frac{A}{A'}$	$\frac{D}{D'}$	$\frac{r'}{r_0}$
1	5.0	1.00	0.67
2	1.0	1.00	1.00
3	1.0	0.10	0.98
4	0.2	0.10	0.90
5	1.0	0.01	0.90
6	0.2	0.01	0.98

See text for details.

FIG. 2.

of integration, so that a larger value of the integral is obtained), we have

$$C \geqq C_s - \int_r^{r'} \frac{1}{r^2}\left(\int_0^r r^2 F(C_s)dr\right)dr.$$

And, completing the integration,

$$C \geqq C_s - \frac{F(C_s)}{6}(r_0^2 - r^2). \tag{24}$$

Hence, if $F(C_s)$ is such that

$$F(C_s) < \frac{6C_s}{r_0^2}$$

C is greater than 0 for any r. That is, if $F(C)$ is such a function that

$$F(C_s) < \frac{6C_s}{r_0^2}$$

for a certain range of values of C_s, then in this range C is greater than 0 for $r \geqq 0$ for any C_s. In the further development this inequality will be assumed for the range of C_s employed. A first approximation may be obtained by assuming $F(C) \ll \frac{6C}{r_0^2}$. (See later.) Then from (24) C is approximately equal to C_s. And, as a second approximation,

$$C = C_s - \int_r^{r_0} \frac{1}{r^2} \left(\int_0^r r^2 F(C_s) dr \right) dr$$

$$= C_s - \frac{F(C_s)}{6} (r_0^2 - r^2).$$

Returning to equation (22), the first approximation gives

$$Q = 4\pi D \int_0^{r_0} F(C_s) r^2 dr$$

$$= \frac{4\pi D F(C_s) r_0^3}{3}.$$

That is, for high C_s, $Q = kF(C_s)$, where $k = \frac{4\pi D r_0^3}{3}$. Using the second approximation,

$$Q = 4\pi D \int_0^r F \left[C_0 - \frac{F(C_s)}{6} (r_0^2 - r^2) \right] r^2 dr.$$

Expanding, assuming C_s large compared with $\frac{F(C_s)}{6} (r_0^2 - r^2)$,

$$Q = 4\pi D \int_0^{r_0} \left[F(C_s) - \frac{F(C_s)}{6} (r_0^2 - r^2) \frac{dF(C_s)}{dC_s} \right] r^2 dr$$

$$= \frac{4\pi D r_0^3 F(C_s)}{3} \left[1 - \frac{r_0^2}{15} \frac{dF(C_s)}{dC_s} \right]. \tag{25}$$

This shows that the effect of diffusion (in lowering C inside the cell and therefore A and Q), represented by the term in brackets, is very small unless $\frac{dF(C_s)}{dC_s}$ is very large.

Experimentally, Q is obtained as a function of C_s. Dropping the subscript, s,

$$Q(C) = kF(C) \left[1 - \frac{r_0^2}{15} \frac{dF(C)}{dC} \right]. \tag{25a}$$

Let $F(C) = \frac{Q(C)}{k} + r_0^2 H(C) +$ higher powers of r_0 times functions of

C. Substituting in (25a)

$$Q(C) = Q(C) + kr_0^2H(C) - Q(C)\frac{r_0^2}{15k}\frac{dQ(C)}{dC}$$

plus higher powers of r_0 times functions of C, or

$$H(C) = \frac{Q(C)}{15k^2}\frac{dQ(C)}{dC}.$$

Hence

$$F(C) = \frac{Q(C)}{k} + \frac{r_0^2Q(C)}{15k^2}\frac{dQ(C)}{dC} + + \cdots$$

$$= \frac{3Q(C)}{4\pi Dr_0^3}\left(1 + \frac{1}{20\pi Dr_0}\frac{dQ(C)}{dC}\right) + \cdots.$$

By Taylor's theorem, neglecting higher powers,

$$F(C) = \frac{Q(C^*)}{k},$$

where

$$C^* = C + \frac{r_0^2}{15k}Q(C);$$

and since

$$A(C) = DF(C),$$

therefore

$$A(C) = \frac{3}{4\pi r_0^3}Q(C^*) \tag{26}$$

and

$$C^* = C + \frac{Q(C)}{20\pi Dr_0}. \tag{27}$$

It will be noted that the larger the value of D, the more nearly do $Q(C)$ and $Q(C^*)$ agree. Q decreases with decrease of C more rapidly than does the true function $A(C)$. This is due to the factor of diffusion, making C at interior regions of the cell lower than C_s. Obviously the closer C approaches C_s, the more nearly will the observed oxygen consumption of the whole cell equal the theoretical consumption per unit volume where this volume has an oxygen pressure of C_s throughout. The higher the diffusion constant and the smaller the radius, the closer will the approach be. The effect of D is apparent in equations (26) and (27), but an increase of radius, r_0, might at first seem to likewise reduce the diffusion factor. When it is recalled, however, that $Q(C)$, the total consumption of a cell, varies with r_0^3, it is clear that the diffusion effect increases as the square of the radius.

For unfertilized sea urchin eggs, Tang (1931) has obtained the data necessary for the calculations. These indicate that the maximal

correction is 0.5 per cent, if the diffusion constant be as assumed. For a constant 100 times smaller, the correction is important, and this case is illustrated in Table III and the resultant $A(C)$ curve

TABLE III

For unfertilized *Arbacia* eggs:
$Q_0 = 6.7 \times 10^{-10}$ cc. O_2 per minute, per egg.
$D = 1.1 \times 10^{-7}$ cc. O_2 per minute, per cm.^{2} per diffusion gradient of one atmosphere per cm. (1/100 Krogh's value assumed).
$C_c = 0.1$ atmosphere.
$r_0 = 3.8 \times 10^{-3}$ cm.

1	2	3	4	5	6	7	8
C_s	$\frac{C_s}{C_c}$	$\frac{Q(C_s)}{\times 10^{-10}}$	$\frac{Q(C_s)}{Q_0}$	C^*	$\frac{Q(C^*)}{\times 10^{-10}}$	$\frac{Q(C^*)}{Q_0}$	$\frac{A(C)}{\times 10^{-3}}$
0.100	1.00	6.7	1.00	0.125	6.7	1.00	2.92
0.090	0.90	6.6 +	0.99	0.115	6.7	1.00	2.92
0.065	0.65	6.6 −	0.98	0.090	6.6 +	0.99	2.89
0.050	0.50	6.4	0.96	0.074	6.6	0.98	2.86
0.035	0.35	6.2	0.92	0.057	6.5	0.97	2.83
0.022	0.22	5.7	0.85	0.044	6.3	0.94	2.74
0.010	0.10	4.4	0.66	0.027	5.9	0.88	2.57
0.004	0.04	3.1	0.46	0.016	5.0	0.75	2.19
0.000	0.00	0.0	0.00	0.000	0.0	0.00	0.00

shown in Fig. 3. For luminous bacteria, because of the very small r_0, the correction turns out to be entirely negligible and the $Q(C)$ curve as observed is also the $A(C)$ curve (Fig. 3).

Columns 1 and 3 are taken from the data on *Arbacia* eggs. These

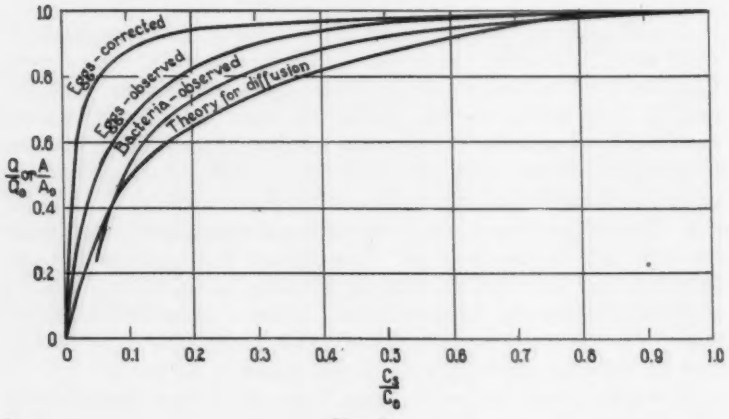


FIG. 3.

values are expressed as ratios in Columns 2 and 4. C^* is calculated by equation (27). $Q(C^*)$ is again obtained by interpolation on the $Q(C)$ curve for eggs, taking Q for each value of C^* as if of C. Column 7 is obtained from 6 and Column 8 from 6 and equation (26). A, as a function of C, is expressed as volume of oxygen per equal volume of eggs per minute.

It may be observed that the assumption made in this last derivation, that $A \ll \frac{6CD}{r_0^2}$ is fully justified for this case. Table IV shows the actual values of each side of the inequality for several values of C.

TABLE IV

C	A	$\frac{6CD}{r_0^2}$
0.1	0.0029	0.480
0.05	0.0029	0.240
0.01	0.0026	0.048
0.004	0.0022	0.019

Examination of Available Data

There are a number of data in the literature for several types of cells sufficiently complete to permit the calculation of the simple C_0; fewer giving as well the observed C_0. Table V summarizes most of this material. It will be noted that the observed C_0 is from 30 to 1000 times greater than the calculated one in all cases except that reported by Warburg and Kubowitz (1929). These workers offer evidence that inadequate shaking at the low oxygen pressures may cause the liquid phase to lag behind the gas phase in oxygen pressure, the dissolved oxygen being used by the cells faster than it can be replaced from the gas phase. Where such a non-equilibrium state obtains, the actual C_0 would be less than supposed, which might account for a reported high C_0. In the experiments of Gerard and Falk (1931), as well as those of Tang (1931), at least, this factor was considered and apparently excluded by controls of the rate of shaking. As pointed out by these workers, wide variation in the conditions of shaking did not affect the C_0. In Amberson's experiments no gas phase was present, only dissolved oxygen being available to the cells from a large volume of liquid. Also, it may be noted that Stephenson and Whetham (1924) found *B. coli* forming lactic acid in air but not in oxygen; and Novy and Soule (1925) observed for the case of tubercle bacilli that growth on the surface of an agar slant was retarded at

TABLE V

Material	Observer	Radius cm. $\times 10^{-4}$	Temp. °C.	Oxygen consumption cu. mm. per minute		C_0 (Atmosphere)	
				per 10^6	per cu. mm.	Observed	Calculated *
Sarcina lutea	Gerard and Falk, 1931	0.63	20	5×10^{-5} 1.7×10^{-4}	0.05 (H_2O) 0.17 (gluc.)	0.01 0.025	1.2×10^{-5} 3.8×10^{-5}
Sea urchin eggs	Tang, 1931 Amberson,† 1928	38	21 20	0.67 3.3	0.004 (unfert.) 0.022 (fert.)	0.10 0.10	7.4×10^{-4} 2.7×10^{-3}
Luminous bacteria	Shoup, 1929 Harvey, 1928	0.6×1.1	21	5×10^{-4}	0.3	0.03	1.9×10^{-5}
Micrococcus candidans	Warburg and Kubowitz, 1929	0.4	10			0.00005	$\alpha10^{-5}$
Yeast	Warburg, 1926	3.5	20		0.2 (gluc. and PO_4)	0.04 < > .20	4×10^{-5}
Amoeba	Pantin, 1930	50×13	15			0.05	5×10^{-4}‡
Nitrifying bacteria	Meyerhof, 1916		35			0.20	
Colpidium	Pitts, (unpublished)	25×15	24	2.5	0.1		6×10^{-3}

* D has been taken throughout as equal to 1.1×10^{-5} (expressed as cc. O_2 diffusing per minute, per $cm.^2$ surface per gradient of 1 atmosphere per cm.)

† For fertilized *Arbacia* eggs, Amberson (1928) finds $C_0 = 0.10$ atmospheres. A cannot be obtained from his data, but he informs me that 3 cc. of a settled egg suspension consumed 0.25 cc. O_2 per hour. If the eggs were just in contact, this would correspond to about 1.5 gm. of eggs, and $A = 0.003$ cu. mm. O_2 per cu. mm. eggs per minute. This value is lower than that obtained by Tang by direct measurement for unfertilized *Arbacia* eggs, and less than 1/7 the corresponding one for fertilized eggs. A similar value for A, 0.002, can be calculated from Warburg's data (1908) on *Strongylocentrotus* eggs, if the same cell radius as *Arbacia* and a specific gravity of 1.05 be assumed.

All agree that fertilized eggs respire five to six times as rapidly as resting ones and the figure used here is 5.5 times that of Tang for the resting cells.

‡ Pantin's calculation.

oxygen pressures below 0.06 atmospheres. In Meyerhof's experiments (1916) also, vigorous shaking is mentioned, and Warburg (1926) found the oxygen consumption of yeast to fall with oxygen pressures below 0.2 atmospheres. On the other hand, despite the careful experimental manipulation described by Warburg and Kubowitz, extra oxygen may have entered their system by diffusion out from the measuring column of Brodie's solution. It is possible that the discrepancy between the results of these experimenters and those of all others is to be attributed to the different cells used.

I am aware of only five sets of observations from which the entire C_e-Q curve can be obtained. The older observations of Amberson (1928) on fertilized *Arbacia* eggs fit almost exactly with those of Tang (1931) on unfertilized ones, even the absolute C_0 values agreeing. This is hardly to be expected with a Q_{O_2} five to six times greater for the fertilized than the resting eggs, and if this identity is confirmed when using the same method on both materials, it will be of considerable importance. Actually, the estimated Q_{O_2} for Amberson's fertilized eggs is less than Tang's value for unfertilized ones (see footnote to Table V), so little more can be said at this time. For bacteria, the curve obtained by Shoup (1929) is available. The complete Q-C_e curve was not obtained in the case of *Sarcina* because of the continually changing Q_{O_2} even in air. Meyerhof (1916, 1917) has presented less complete data for the nitrifying bacteria. All

TABLE VI

$\frac{C_e}{C_0}$	$\frac{Q}{Q_0}$			
	Arbacia eggs	Luminous bacteria	Nitrite formers	Nitrate formers
1.00	1.00	1.00	1.00	1.00
0.90	0.99	0.98	0.99	0.98
0.65	0.98	0.95	0.97	0.88
0.50	0.96	0.92	0.92	0.80
0.35	0.92	0.86	0.79	0.71
0.22	0.85	0.74	0.54	0.59
0.10	0.66	0.53	0.24	0.34
0.04	0.46	0.15	(.13)	
0.00	0.00	0.00	0.00	0.00

these data are summed up in Table VI. The oxygen consumed by each type of cell is expressed as a fraction of the maximum for that cell, and all are aligned against C_e values expressed as fractions of C_0, this being likewise taken as unity for all.

It is especially interesting to compare these observed curves with those calculated on the various types of assumption. For the luminous bacteria, the observed curve agrees fairly well with the one calculated on the assumption that there is no concentric zoning of the cell and that oxygen consumption is independent of pressure, except as diffusion becomes inadequate. Q falls with C_e, in other words, as would be expected if the only factor involved were the depth of penetration. Still the absolute value of C_0 as measured is far above that expected on the grounds of the diffusion equation. Either, therefore, the diffusion coefficient for oxygen through the bacterial cell is over 1,000 times smaller than that for fascia and muscle,[2] or the fall of Q with C_e is quite independent of any diffusion factor.

In the case of sea urchin eggs, the situation is still more complicated. The observed curve does not fit the simple calculated one. It does fit, however, a curve calculated on the assumption that A is independent of C but including the complication that A is not uniform in all cell regions but is greater in a cortical zone than in the central portion (Curve I, Fig. 2, and Fig. 3). A lower diffusion constant in the cortical zone can not yield this shape of curve.

Again, the absolute value of C_e, 100 times smaller than that calculated, makes it very difficult to interpret the agreement with the curves calculated on a diffusion basis. If oxygen diffuses through sea urchin eggs 100 times more slowly than through muscle, all the results become quantitatively intelligible. But it is more probable that failing diffusion is only a very minor factor, even in these larger eggs, and that the direct fall of A with falling C is the primary one.

Assuming that $A(C)$ is directly given by the $Q(C)$ curve in the case of luminous bacteria and is obtained by correcting for diffusion in the case of *Arbacia* eggs, we are left with an empiric relation between oxygen concentration and rate of oxidations. Shoup (1929) offers a physical interpretation of his results in terms of adsorption of oxygen on or evaporation from surfaces in the cell. He puts Langmuir's (1916) equation for such gas adsorption in the form:

$$k_1 p(1 - \theta) = k_2 \theta$$

where k_1 and k_2 are adsorption and evaporation constants respectively and θ is the fraction of the total surface covered, and indicates that this will fit his curve if θ be assumed to represent the percentage of maximal respiration, *i.e.*, $\frac{A}{A_0}$ (A_0 being the rate of oxidation at oxygen

[2] Each bacterial cell, as any small particle, is surrounded by an envelope of fluid which is essentially not stirred. This increases the effective radius of the cell and hinders diffusion. This alone, however, to increase C_0 1,000 times, would require a layer over 300 times the radius of the cell. (See Equation 7.)

pressure C_c; A the rate at pressure C). Warburg and Kubowitz (1929) also developed a relationship in terms of reaction velocities for oxidation and reduction of an iron catalyst.

A somewhat similar development follows.

It may be safely assumed that oxygen combines with at least one intermediate catalyst before acting upon the substrate. Then, neglecting any details,

$$O_2 + \text{Int.} \rightarrow \text{Int.}O_2$$
$$\text{Int.}O_2 + \text{Sub.} \rightarrow \text{Sub.}O_2.$$

For an equilibrium state, the concentration of $\text{Int.}O_2$ must remain constant and the rates of its formation and reduction must be equal. So

$$[O_2][\text{Int.}]k_0 = A = [\text{Int.}O_2][\text{Sub.}]k_r, \tag{28}$$

where A is the velocity of combination of oxygen, or rate of respiration, and k_0 and k_r constants of oxidation and reduction.

In the presence of an excess of substrate, [Sub.] may be assumed to be a constant, k_s, and

$$A = [\text{Int.}O_2]k_rk_s.$$

The maximal rate of respiration, A_0, will be obtained when all Int., Int._t, is in the oxidized state; and the respiration as a fraction of the maximum is then

$$\frac{A}{A_0} = \frac{[\text{Int.}O_2]}{[\text{Int.}_t]}. \tag{29}$$

From (28)

$$\frac{[\text{Int.}]}{[\text{Int.}O_2]} = \frac{k_rk_s}{k_0[O_2]} = \frac{K}{[O_2]}.$$

Adding 1 to each side and inverting,

$$\frac{[\text{Int.}O_2]}{[\text{Int.}] + [\text{Int.}O_2]} = \frac{[O_2]}{K + [O_2]}.$$

But

$$[\text{Int.}] + [\text{Int.}O_2] = [\text{Int.}_t].$$

So that

$$\frac{A}{A_0} = \frac{[O_2]}{K + [O_2]}.^3 \tag{30}$$

[3] If [Sub.] is not assumed to be constant, the final equation is

$$\frac{A}{A_0} = \frac{[O_2]}{K[\text{Sub.}] + [O_2]}.$$

When [Sub.] is maximum, the effect of $[O_2]$ is greatest; as [Sub.] becomes less, it, rather than $[O_2]$, becomes the critical factor.

But Langmuir's equation may be readily put in an identical form by using the notation $[O_2]$ for p, $\frac{A}{A_0}$ for θ, and K for $\frac{k_2}{k_1}$. Obviously, an agreement between the curve of this equation and that obtained experimentally fails to substantiate either of the assumed mechanisms as the one underlying these oxidations. As a matter of fact, the experimental data do not agree too well with the equation.

Shoup's data (as read from his curve) yield the following:

Per cent O_2	$\frac{A}{A_0}$	K
3.00 and over	1.00	0.00
1.32	0.90	0.14
0.79	0.80	0.20
0.55	0.70	0.23
0.38	0.60	0.25
0.27	0.50	0.27
0.22	0.40	0.33
0.17	0.30	0.40
0.13	0.20	0.52

It is sufficiently obvious that K is even roughly constant only in the middle range.

Tang's data, similarly handled, give:

Per cent O_2	$\frac{Q}{Q_0}$ $\frac{A}{A_0}$	K
10.0 and over	1.00	0.0
9.0	0.99	0.1
6.5	0.94	0.3
5.0	0.87	0.8
3.5	0.78	1.0
2.2	0.66	1.1
1.0	0.49	1.0
0.4	0.27	1.1
0.0	0.00	—

The agreement here is somewhat better. K is approximately constant up to 80 per cent maximal respiration.

For the nitrifying bacteria, the curves of the nitrite and the nitrate formers are widely different, and both are far from agreeing with the derived equation.

It seems to follow, clearly enough, that the Q—C_s curve is far from identical for the various cells, and that the individual form is probably but little dependent on the limits set by diffusion. No one

	NO_2		NO_3	
Per cent O_2	$\frac{Q}{Q_0}$	K	$\frac{Q}{Q_0}$	K
20 and over	1.00	0.0	1.00	0.0
15	0.99 +	0.1	0.92	1.3
10	0.98	0.2	0.80	2.5
7	0.92	0.7	0.72	7.0
4	0.68	1.9	0.57	4.0
2	0.35	3.7	0.34	3.9

simple assumption as to oxidizing mechanism appears able to fit all cases. Additional data for a much greater variety of cells are greatly to be desired, and must precede any more elaborate attempt to theoretically derive the important $A(C)$ curve.

Further important questions concern the change in Q_0 attending changed conditions. The fertilized sea urchin egg, for example, after a tremendous initial burst of oxidation, continues to use oxygen at five to six times the resting rate. Is this to be interpreted in terms of a sudden increase of available Int.? None the less, so far as present data show, there is no change in C_0 or in $A(C)$. *Sarcina*, on the other hand, shows an increase in C_0 approximately in proportion to the increased Q when glucose is added. The complicated relations between lactic acid and Q_0 have been previously discussed (Gerard, 1931). The suddenly increased respiration of muscle, nerve, and other cells associated with activity also comes to mind in this connection.

Finally, it is to be noted that most cells exhibit a considerable "factor of safety" in their rate of energy liberation. The respiration may be very considerably depressed by low oxygen pressures before the lessened energy interferes with the physiological activity of a cell. Thus luminous bacteria do not begin to dim until the oxygen is reduced to 0.25 per cent (Shoup, 1929), when respiration has been reduced to half, and still luminesce at 0.0007 per cent O_2 (Harvey, 1928). *Arbacia* eggs show no disturbance in cleavage at oxygen concentrations over 1.5 per cent (Amberson, 1928) though 10 per cent is required for maximal respiration.

Similar relations are difficult to demonstrate for tissues because of the tremendously greater importance of diffusion in the larger masses. For the case of nerve, it was shown (Gerard, 1927) that the C_0 calculated from the simple diffusion equation, assuming A independent of C, was in good agreement with the observed value. It is probable, however, that more careful examination of this point would reveal

some dependence of A on C. Less direct evidence indicates that here, also, conduction is possible when the energy available is but a fraction of the normal amount. (Gerard, 1930).

Summary

The question of diffusion of oxygen into cells, in distinction to tissues, requires examination of several factors not previously considered. The relation between external oxygen pressure and rate of respiration may be complicated by the existence of different diffusion constants and respiration rates in separate cell zones.

Equations are developed covering such cases for two concentric zones in a sphere and relating rate of respiration to oxygen concentration, cell radius, and diffusion coefficient.

When the assumption, made in similar studies for tissues, that consumption in any one region is independent of oxygen pressure if above zero, is avoided, the observed consumption-pressure curve can be corrected for diffusion effects to give the true consumption-pressure relation. Equations for this correction are presented.

Available data on bacteria, marine eggs, and other material, are examined quantitatively. It appears very probable that oxygen consumption in each region is dependent on oxygen concentration up to quite considerable values. The theoretical bases for such a dependence are discussed.

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THE EFFECT OF TEMPERATURE CHANGES UPON THE PULSATIONS OF ISOLATED SCALE MELANOPHORES OF *FUNDULUS HETEROCLITUS*

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When Spaeth (1916*a*) published a method for producing pulsations in the isolated scale melanophores of *Fundulus heteroclitus* he greatly broadened the possible scope of investigations dealing with the direct action of various physical and chemical factors upon such preparations. Recognizing this fact, it was thought that perhaps an investigation of the effects of temperature changes upon such pulsations would help in extending our knowledge of how heat and cold act upon the pigment cells. The melanophores of isolated *Fundulus* scales will respond directly to variations in temperature (Spaeth, 1913; Smith, 1928), heat causing a contraction and cold an expansion of the pigment granules. But beyond these descriptive facts our knowledge does not go. One's interest is, therefore, directed towards ascertaining how these melanophores, when induced to pulsate through the use of Spaeth's method, would react to temperature changes, with the hope of perhaps obtaining information permitting a more exact analysis of this phenomenon.

Briefly, Spaeth's method of producing pulsations is as follows: First the isolated *Fundulus* scales are immersed in N/10 $BaCl_2$ for 5 minutes, in which time their melanophores become punctate; then they are transferred to N/10 NaCl, where within fifteen to twenty minutes after their removal to this solution, the pulsations become evident. These pulsations take the form of periodic migrations of the pigment granules in and out of the processes of the melanophores, the movements of the individual pigment granules being readily seen with ordinary microscopic magnification. Hence, we have a preparation which, in its rhythmical activity, is comparable to the behavior seen in other effector organs such as cilia, heart muscle, and smooth muscle. To this add the advantage that in the melanophore we deal with an effector consisting of only a single cell. In studying the effects of temperature changes upon pulsating pigment cells, our interest is therefore centered on how this factor may alter either the rate or the extent of these rhythmical migrations, distal and proximal, of the pigment.

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Now it is well known that the color changes exhibited by numerous fishes in response to variations in the tint of the background over which they swim are determined in a large measure by the behavior of these melanophores and allied pigment cells. However, in the intact fish the melanophores do not pulsate; such alterations as take place in the distribution of their pigment are relatively slow in occurrence and only in response to a change in the color of the background or of the incident light intensity. Therefore, in dealing with pulsating isolated melanophores, we are not concerned with a phenomenon which plays a part in the natural economy of the animal.

As a cell the melanophore is a relatively large structure with numerous branching processes exceeding in length by three or four times the diameter of the central body from which they ramify. These processes, when empty of pigment, are transparent under ordinary conditions of illumination. In fact, the visible manifestation of the shape of the cell is due entirely to the pigment granules contained within itself, and if this pigment is concentrated in the central body, the cell appears to be spherical. The term punctate is applied to melanophores in this state. But when under the proper circumstances these invisible processes are invaded by a distal migration of the pigment granules, their outlines are rendered visible. It is this migration of the pigment in and out of the otherwise transparent processes that creates the illusion of a change in the shape of the melanophore. The use of the terms "contraction" and "expansion" of the melanophores must, therefore, be considered as only convenient expressions describing a redistribution of the pigment granules within the melanophores.

An investigation of this nature requires an apparatus sensitive enough to accurately measure these pulsatory movements within the processes of the melanophores. Such an apparatus was modeled with some modifications after one originally devised by Spaeth (1916*b*) for the same purpose, and employed by him in connection with his researches upon the effects of electrical stimulation upon the isolated melanophore. As my investigations were not concerned with the effects of electrical stimulation, but were rather studies of the effects of temperature variations, certain changes were necessarily introduced in the original design, which is fully described and pictured elsewhere (Spaeth, 1916*c*); the most important among these changes being the addition of a warm stage to the set-up. This was done by fitting to the stage of the microscope a water bath in which a small Stender dish could be almost completely submerged. This Stender dish was filled with N/10 NaCl, and in this solution the scales containing the pulsating melanophores were placed. By altering the temperature of the water

flowing through the bath, it was also possible to change the temperature of the NaCl. Any desired temperature between 0° and 45° C. could be attained within two minutes in the Stender dish and maintained indefinitely with no variation greater than 0.2° C.

To measure the pulsations and to make a permanent record of their variations, the microscope was equipped with an adjustable micrometer ocular in place of the usual eye piece, the adjusting screw of the ocular being fitted to a pulley operating a lever capable of writing upon a kymograph drum. In making a record, a scale was placed under the objective, and one of its pulsating melanophores selected for observation, a convenient division of the ocular scale being brought level and at right angles to the apparent end of one of the pulsating processes of this selected melanophore. As the pigment granules in this process migrated either distally or proximally, the ocular scale division kept pace with those granules which marked the apparent distal boundary of the process. Such action was accomplished by turning the adjusting screw of the movable ocular scale. To insure accurate measurements, it was absolutely necessary that the melanophore be kept motionless throughout the experiment. This was accomplished first by bringing a microscope clamp, attached to the warm stage, to bear lightly upon the end of the scale, care being taken that the melanophores were not damaged or stimulated by too great a pressure. Since such a contingency was always imminent, this method for holding the scale was eventually discarded in favor of another. The new method consisted in laying a paraffine mould on the bottom of the Stender dish cut so as to accommodate the scale. In this way the scale was snugly secured without being subjected to any noticeable pressure. No effect of the paraffine upon the pulsations could be demonstrated. As the adjusting screw was turned to follow the migrations of the distal pigment granules, the pulley attached to it moved the lever in contact with the kymograph drum up and down. Consequently, with the lever writing upon the smoked kymograph paper, a permanent and accurate record of the pulsations could be obtained. The speed of the drum was adjusted to one revolution in approximately forty-five minutes.

Records of the melanophore pulsations were not made until the cells had been exposed to NaCl for at least one hour subsequent to their immersion in $BaCl_2$. While pulsations are first evident in fifteen to twenty minutes after the removal of the scales from $BaCl_2$, it usually requires an additional thirty minutes before they become constant in extent and frequency at any given temperature. The first pulsations are extremely slight, but once started they gradually become more pronounced until they finally reach the maximum characteristic for

the particular temperature in question. An hour, then, gives ample time to insure the attainment of this point. However, once this maximum was reached, a new state of equilibrium incident upon a change in temperature could be attained within 5 minutes. Therefore, a record having been made at a certain temperature, 10 minutes being usually sufficient for this purpose, another record at another temperature could be made 10 minutes after the change in temperature was effected. The extent of the temperature change between observations was from 1° to 10° C., the usual variation being about 5° C. The experiments were begun at either end of the temperature scale, and by a series of four or five steps, the temperature was raised or lowered to the opposite extreme, the result being the same regardless of whether the initial record was made at a high or a low temperature. In this way one melanophore was kept under continuous observation for two or three hours. At the beginning, a single process of a single melanophore was selected, and the record made from the pulsations of this process. The numerous melanophores of a single isolated scale vary considerably in size. Though an attempt was made to confine the experiments to melanophores approximately equal in size, this was not always possible. However, such attempts were more or less nullified by the fact that it was impossible to predict the maximal length of a process by its appearance when the cell was in an almost punctate or even semi-expanded condition. As fully one-half the experiments were begun under such circumstances, certain corrections in the data were necessary before the results could be subjected to satisfactory analysis.

Temperature Changes and their Effect upon the Extent of the Pulsations

On analysing the results obtained from the records pertaining to the relationship between the temperature and the amount of extension of the melanophore processes during their pulsations, two general facts emerge: (1) An increase in temperature causes a decrease in the extent of the pulsations and (2) this relationship between temperature and extension is a rectilinear one. Table I presents the data relative to this relationship. The figures given in the temperature columns represent the average of the mean maximal and minimal lengths of a single selected melanophore process during its pulsations at a given temperature, obtained by measuring in centimeters the height of the highest and lowest points of the curve traced on the kymograph records. This averaging was necessary for two reasons: first because during the pulsations the maximal extensions of a single process were not

TABLE I

Average Migration

C.	9.5°	12°	13°	13.5°	14°	14.5°	15°	15.5°	16°	16.5°	17°	17.5°	18°	18.5°	19°	19.5°	20°	20.5°	21°	21.5°	22°
1				11.5												8					
2							38.5					34.5			30						
3	38	38					32		25.5		13.5										
4											34					25					
5																		16			
6												32									
7																					
8											28						25				
9																				27	
10																					
11									11								7				
12							25.5								18.5						
13			34										23								
14							25										9				
15									37												
16									18.5											11	
17								21									18.5				
Av.							30.2		23		25						15.0				

TABLE I (*Continued*)

Average Migration

C.	22.5°	23°	23.5°	24°	24.5°	25°	25.5°	26°	26.5°	27°	27.5°	28°	28.5°	29°	29.5°	30°	30.5°	31°	31.5°	32°
1							4								1					
2	16.5							10												
3																				
4	17				12															
5	13					11.5					6.5									
6					23								8.5						1.5	
7	39			27				17				6.5								0
8		17.5						6.5						1						
9	23			18			18				8				5			1.5		
10				23					18							8.5				
11		9										2								
12		14.5						10						6						
13	14						12								8					
14	6.5							4.5												
15						19		12			8.5									
16						5.5										1				
17				13					3.5					.5						
Av.	18.4	14.0		20.0		12.0	11.3	10.0			7.7			2.5	4.7					

always constant at a given temperature, a fact which applies equally to the minimal extensions, and secondly because at a point two or three degrees above the lowest temperature at which pulsations occurred (about 12° C.), the process would attain during its period of greatest extension its maximal possible length. However, over these same low temperatures the length of the process during the phase of minimal extension was greater, the lower the temperature, the minimal extension gradually approaching the maximal extension as the temperature came nearer to the critical point. Similarily, as the temperature rose to within two or three degrees of the upper limit (about 32° C.) at which pulsations stopped, the melanophores would appear punctate during their phases of minimal contraction, though at these temperatures the process would keep on periodically extending; the amount of extension, however, decreasing as the temperature rose. On reaching the upper limit all pulsations ceased and the cells remained permanently punctate. Therefore, by using averages of the maximal and minimal lengths, figures are obtained which at the extreme temperatures vary in accordance with the temperature changes. This, of course, necessitates treating the data obtained in the middle of the temperature scale in the same manner.

As indicated in Table I, whenever observations were made upon three or more different processes at the same temperatures, an average of the figures expressing the relative amount of extension was made. These averages are given in the bottom line of the table and are plotted with the result shown in Fig. 1. From this curve it is clear that as the temperature increases, the extension of the melanophore processes during their pulsations decreases; a result to be expected, since it was previously known that high temperatures, when they affect the melanophores directly, cause a withdrawal of the pigment into the central body. Furthermore, it is quite clear from Fig. 1 that the relationship between temperature and extension is a rectilinear one.

The observational data given in Table I represent the true relative lengths of the melanophore processes as they were calculated from the kymograph records. It is apparent, from an inspection of the table, that there is considerable variation in the pulsatory activity among the different processes in regard to the amount of extension shown at the same temperature. Because of this variation, the figures cannot be profitably compared unless they are all corrected on the assumption that the amount of extension in any one process at 20° C. is equal to twenty. When this is done and the results plotted as shown in Fig. 2, it is obvious that the effect of temperature changes on all pulsating melanophore processes is virtually the same so far as any relative alter-

ation in the amount of extension is concerned. The one exception is case 3 of Table I, where the corrected figures were so at variance with the others that it was impossible to plot them upon the curve. They have consequently been omitted from Fig. 2. But aside from

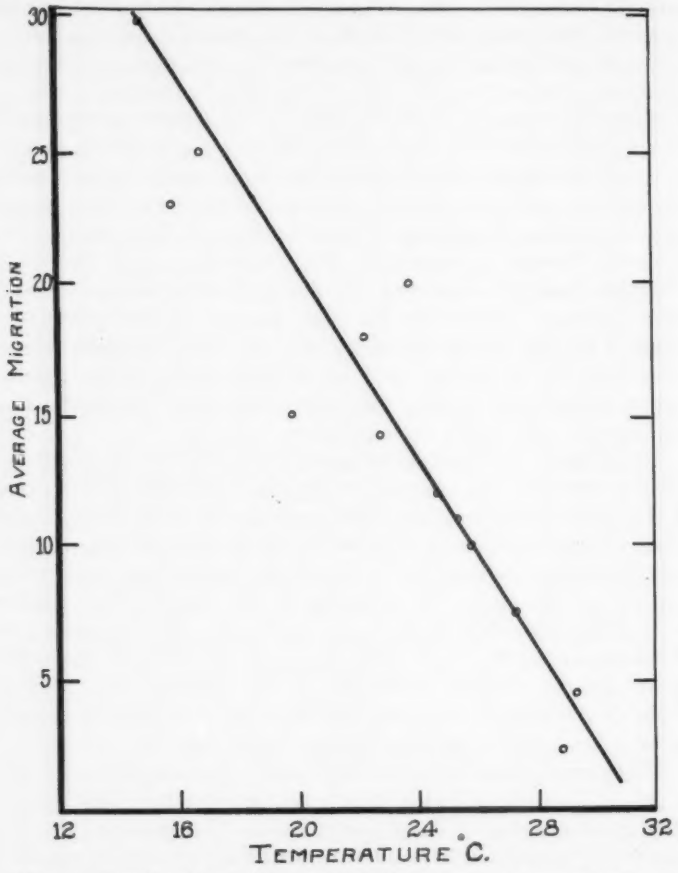

FIG. 1. Showing the rectilinear relationship between the temperature of the medium (abscissæ) and the average length of a melanophore process during a single pulsation (ordinates). The points plotted are those given in the average column of Table I.

this unexplained discrepancy, the curve substantiates the conclusions drawn from the one given in Fig. 1.

In the higher reaches of the temperature scale pulsations cease

between 30° and 34° C., the average being approximately 32° C. When this occurs, the melanophore assumes a permanent punctate state. At low temperatures, however, the melanophore comes to rest with all of its processes maximally extended, the pigment being evenly, if thinly, distributed throughout the whole length of the process. Such a condition is generally attained at about 12° C., slight move-

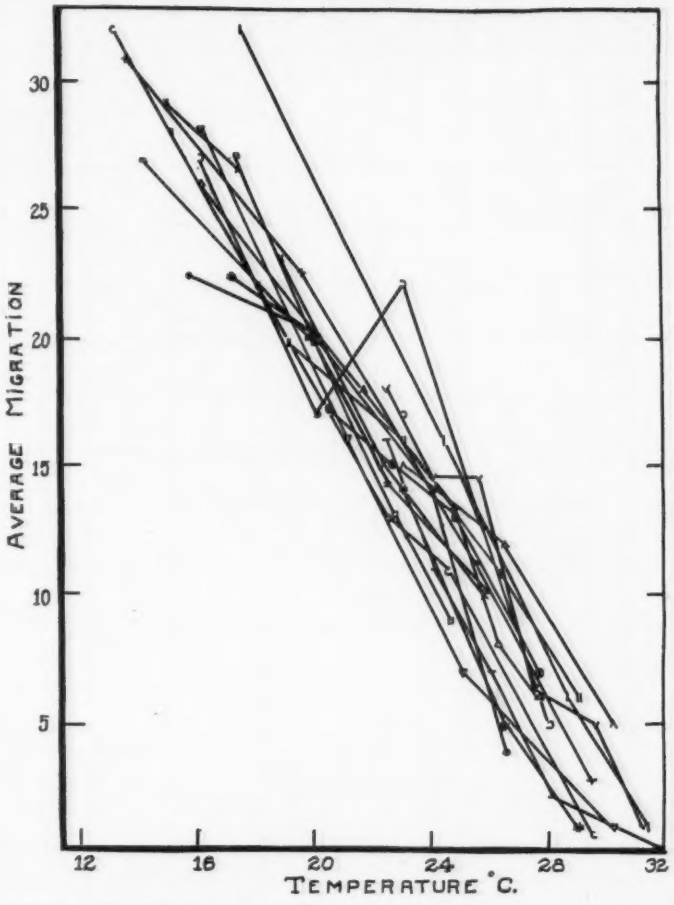

FIG. 2. Showing the result of plotting seventeen different experiments on the relationship between the temperature of the medium (abscissæ) and the average length of the melanophore process during a single pulsation (ordinates); the average length of the melanophore process being taken in all cases as equal to 20 at 20° C., the data in Table I being corrected accordingly.

TABLE II

Pulsations per Minute

C.	9.5°	12°	13°	13.5°	14°	14.5°	15°	15.5°	16°	16.5°	17°	17.5°	18°	18.5°	19°	19.5°	20°	20.5°	21°	21.5°	22°
1				.6												1.8					
2							.7					.52			.85						
3	.5						.9		1.1		1.35										
4											.8					1.2					
5																		.4			
6												.4									
7																					
8											.8						.9				
9																					
10																				1.3	
11									.8								1.2				
12							.8								1.5						
13			.2										.4								
14							.4										.9				
15									.8												
16									1.3											1.8	
17								.3									.4				
Av.							.7		1.0		.98						.85				

TABLE II (*Continued*)

Pulsations per Minute

C.	22.5°	23°	23.5°	24°	24.5°	25°	25.5°	26°	26.5°	27°	27.5°	28°	28.5°	29°	29.5°	30°	30.5°	31°	31.5°	32°
1							2.5								3.0					
2	1.4							2.0												
3																				
4	1.9				2.1															
5	.7					1.1					1.8									
6					.6								1.7						1.1	
7	1.1			1.3				1.7				2.4								0
8		1.0						1.5						1.2						
9	1.2			2.1			1.8				2.1				2.2			1.8		
10				1.6					1.8							2.3				
11		2.1										5.0								
12		4.0						4.4						1.1						
13	.9						1.3								2.4					
14	1.2							2.4												
15						2.0		2.8			3.4									
16						3.7										8.0				
17				1.0					1.4					.8						
Av.	1.2	2.3		1.5		2.27	1.87	2.46			2.43			1.03	2.53					

ments persisting in some cells until 10° C.; while others come to rest at temperatures as high as 14° C. In many cases Brownian movement was observed among the pigment granules in all parts of the process, even though the pulsations had entirely ceased.

Temperature Changes and their Effect upon the Frequency of Pulsations

On considering the action of temperature changes upon the frequency of pulsations, we find (1) that an increase in temperature from 12° C. to within the neighborhood of 27° C. causes an increase in the rate of pulsations, which is followed by a rapid decrease as the temperature mounts higher, and (2) that the relationship between temperature and the increase in rate during the rise from 12° to about 27° C. is a non-rectilinear one. The data obtained from an analysis of the kyomograph records are presented in Table II, where the figures given in the columns under the temperature headings show the absolute number of pulsations of a single melanophore process in one minute at the temperature indicated. Every figure in the temperature columns represents the pulsations per minute of a different process, no two processes being attached to the same melanophore and every melanophore being selected from a different scale. Between different cells the variation in the frequency of the pulsations at the same temperature is considerable, as shown in Table II; at 22.5° C., for example, a process of one cell shows 0.7 pulsation per minute, while a process of another cell shows 1.9 pulsations per minute. In every case where three or more observations were made at the same temperature, the results were averaged, the averages being given in the bottom line of the table.

In Fig. 3 these averages are plotted, such plotting bringing out the previously-mentioned increase in rate of pulsation during the greater part of the temperature rise, the relationship in this case, in contrast to the relationship between temperature changes and the extent of the pulsations, being clearly non-rectilinear in nature. The possible significance of this will be discussed later. From this curve the temperature coefficient of the melanophore pulsations can be calculated, though such coefficients can have only an approximate value. Between 15° C. and 25° C. the temperature coefficient is 3.57, while between 20° C. and 30° C. it is 3.6. It is doubtful whether the difference between the two is significant, especially as the latter coefficient can have only a speculative value, owing to the irregular appearance of the depressant effect upon the rate of pulsations at the warmer end of the temperature scale.

As there is a wide variation in the behavior of different processes at the same temperature, the data presented in Table II must be corrected before they can be presented in intelligible graphic form. In doing this it was arbitrarily assumed that the rate of pulsation of all melanophore processes at 20° C. was equal to one, the necessary corrections for all other temperatures being made on this basis. These

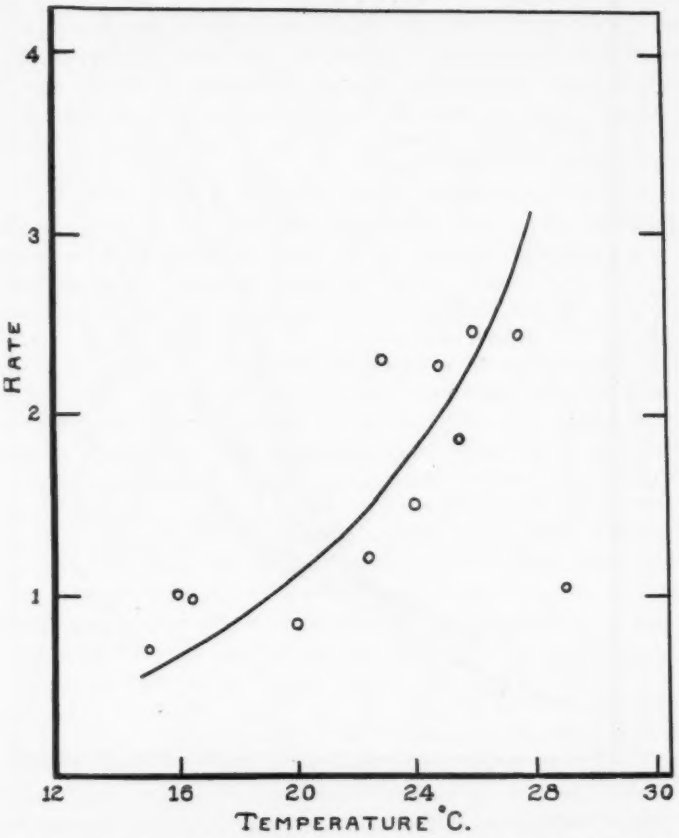

FIG. 3. Showing the non-rectilinear relationship between the temperature of the medium (abscissæ) and the number of pulsations per minute (ordinates). The points plotted are those given in the average column of Table II.

corrected data were then plotted as shown in Fig. 4, the curve so obtained substantiating the relationship expressed in Fig. 3. Furthermore, the points of Fig. 4 group themselves closely about the curve given in Fig. 3.

As to the point where the frequency of pulsations decreases with further increases in temperature, there is considerable variation among the different melanophores, some showing no diminution in the in-

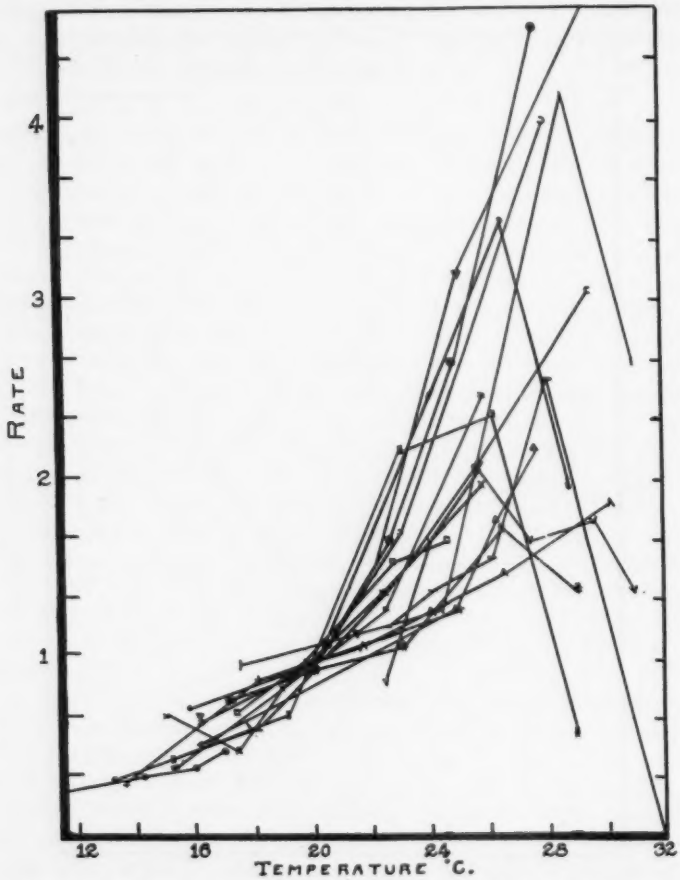

FIG. 4. Showing the result of plotting seventeen different experiments on the relationship between the temperature of the medium (abscissæ) and the number of pulsations per minute (ordinates); the number of pulsations per minute being taken in all cases as equal to one at 20° C., and the data in Table II corrected accordingly.

crease in rate at temperatures as high as 30° C., while in others this depressant effect is initiated at temperatures as low as 25° C. In general, this point occurs in the neighborhood of 27° C. Once a

decrement in rate is established, a further rise in temperature produces a fall, which is very rapid in comparison to the relatively slow increment which preceded it, the difference being graphically shown in Fig. 4. The exact course of this reduction in rate is uncertain, as the data at hand do not permit an accurate analysis.

Discussion

A rise in temperature, then, affects the pulsating pigment granules of an isolated *Fundulus* scale melanophore by increasing the frequency of their pulsatory movements up to a certain point and at the same time reducing the distance migrated by these granules during their pulsations, which in effect gives the appearance of a shortening of the melanophore process. Gray (1923) mentioned a somewhat similar situation in his study of the action of temperature changes upon ciliary activity when he observed a progressive reduction in the amplitude of the ciliary beat between 34° and 38° C., though the frequency of the beat increased in proportion to the rise in temperature. In Gray's experiments, however, this reduction in amplitude was seen only above 34° C. Between 0° and 34° C. the amplitude of the beat remained constant, though the frequency increased as the temperature became higher. One must, therefore, make a distinction between cilia and melanophores in this respect, for in the latter any temperature rise capable of increasing the frequency of the pulsation also produces a corresponding decrease in the extension of the granules during these pulsations. Gray suggests that in his experiments the decrease in the amplitude of the ciliary beat above 34° C. might be explained by assuming "that at this temperature the rate at which the process of contraction is induced in the cilium is more rapid than the process of relaxation, so that relaxation begins before contraction is complete."

The decrease in apparent extension of the melanophore process with an increase in temperature might also be explained in the same way. Once a distal migration of the pigment granules has been established, there is the possibility of its continuing until either factors are brought into play, producing a movement in the opposite direction, or until the granules reach the end of the process and are incapable of further migration. But except with extreme low temperatures, proximal pigment migration sets in before the limits of distal migration are reached. Therefore, the factors producing this proximal migration are capable of overcoming those governing the distal migration, or else the latter cease to operate before the pigment granules reach the limits of the process. But whichever explanation is correct, the matter is of secondary importance, as the factors determining the extent of

the migration of the pigment granules must be independent of both. This conclusion is deducible from the response of melanophore pulsations to variations in temperature, for such variations seem to influence independently two separate series of reactions within the pigment cell, one governing frequency, and the other the extent of the migration. The fact that the distance migrated by the pigment granules during their pulsations shows a rectilinear relationship while the frequency of the pulsations varies in a non-rectilinear manner in relation to temperature changes hardly warrants any other interpretation. Therefore, if extension is determined by frequency, and it is by no means clear that this is so, it is only because the factors leading to a proximal migration dominate those which might favor further extension and not because extension is a function of frequency. It is interesting to note in this connection that Clark (1920) demonstrated in the isolated rabbit's auricle a rectilinear decrease in the height of contraction with an increase in temperature; and at the same time observed a non-rectilinear increase in the frequency of the beat. In isolated frog's heart this was not the case, both the diminution in the force of contraction and the increase in frequency of the beat caused by a rise in temperature being of a non-rectilinear nature.

Since melanophores contract in the presence of an insufficient amount of oxygen, there may exist a relationship between the decrease in the oxygen tension of the water with an increasing temperature and the decrease in extension of the melanophore processes. But Spaeth (1913) has shown that in the isolated scale, melanophore contraction of the pigment occurs at high temperatures both in media furnished with an excess supply of oxygen and in media with the usual oxygen tension for the temperature in question. Therefore, the contraction of the pigment granules with an increase in temperature cannot be due to a lack of oxygen. But the contraction of pigment in a non-pulsating melanophore may be governed by factors other than those which control the amount of extension of the pigment granules in a pulsating melanophore. Unfortunately this question is left open as my observations did not include the effect of a decrease in oxygen supply upon either the frequency or the extent of pulsations and nothing is known concerning this question from other sources. The sudden decrease in the frequency of pulsations with temperatures above 27° C., following a consistent increase with a rise in temperature to this point, may be concerned with a possible inadequacy in the oxygen supply to the melanophores at high temperatures. Whether or not this is true for pulsating melanophores, it is apparently untrue in the case of cilia, for, according to Gray (1923) the oxygen consumption of the tissue

keeps pace with the speed of the beat over the temperatures used in his experiment. As a consequence of this, Gray doubts whether a rise in temperature involves in well-ventilated tissues a lack of oxygen except with very high temperatures, when a decrease in mechanical activity might well be due to insufficient oxygen.

The effects of temperature changes upon pulsating melanophores and the effects of temperature changes upon ameboid movement, ciliary activity, and heart beat show a certain similarity. In all cases, the effect is one of an increase in frequency, an increase which is more rapid than a rectilinear function. But while the temperature coefficient of ameboid movement, ciliary activity, and heart beat are all in fairly close agreement, the melanophore is peculiar in that it has a temperature coefficient which in comparison to the others is remarkably high. As we have previously seen, the temperature coefficient of a pulsating melanophore is 3.57 between 15° and 25° C., and 3.6 between 20° and 30° C., figures which can be compared with those given in Table III.

TABLE III

Q_{10}	15°–25° C.	20°–30° C.
Ciliary activity	2.15	1.95 (Gray, 1923)
Ameboid movement	2.04	— (Pantin, 1924*b*)
Heart beat	2.10	1.90 (Clark, 1920)
Melanophore pulsations	3.57	3.60

A determinative rôle on the part of viscosity changes has already been assigned as a possible factor controlling the movements of pigment granules within the melanophores and it is easy to imagine the same factor assuming considerable importance in governing the frequency and the extent of melanophore pulsations. But unfortunately our knowledge of the exact effect of temperature changes upon the viscosity of protoplasm is at present too meager and too contradictory to warrant any extended conclusions as to how much viscosity is involved in responses of melanophore pulsations to variations in temperature. Both Pantin (1924*a*) and Heilbrunn (1924) have interested themselves in the relationship between temperature and its effect upon animal protoplasm, but with no agreement as to results. Pantin, who worked with the protoplasm of *Nereis* eggs, states that as the temperature increases, the viscosity decreases, the relationship being a non-rectilinear one with a temperature coefficient of 1.3 between 15° C. and 25° C. and 1.26 between 20° C. and 30° C. Heilbrunn, on the other hand, working with *Cumingia* eggs found a sharp decrease in viscosity between 0° C. and 2° C., followed, as the temperature rose further, by a gradual increase which came to a maximum in the neighborhood of

16° C. This in turn was replaced by a decrease until the temperature reached about 30° C., when within the next degree or two there was a very rapid increase. But on the basis of either Pantin's or Heilbrunn's work it is clear that viscosity is not an important factor in determining the responses of melanophore pulsations to temperature changes. The difference between the temperature coefficients which Pantin obtained and those found to apply to melanophore pulsations are so great that the viscosity changes in the melanophores can hardly be of importance in governing the variations in pulsations associated with temperature changes. While, on the basis of Heilbrunn's results, if viscosity changes within the melanophore were an important factor, the curves obtained in these studies on the relationship between temperature changes and the amount of extension of the pigment granules together with the frequency of their pulsations would hardly be as regular as they are, but would show significant variations or breaks in accordance with the curve obtained by Heilbrunn. For the present at least, the conclusion seems inevitable that viscosity changes in the protoplasm of the melanophores attendant upon variations in temperature are not responsible for the particular manner in which the pulsations of these cells respond to alterations in temperature. Whether viscosity is of importance in governing the non-pulsatory changes which occur in the distribution of melanophore pigment, as a fish adapts itself to shifts in the color of its background, is quite possibly another matter and one about which nothing can be stated here.

Summary

1. Pulsations in isolated scale melanophores of *Fundulus heteroclitus* occur in N/10 NaCl after treatment with N/10 $BaCl_2$ at any temperature between 12° and 32° C.

2. Below 12° C. pulsations cease, the melanophore coming to rest in the maximally expanded state.

3. Above 32° C. pulsations also cease, the melanophore coming to rest in the punctate state.

4. Between 12° and 32° C. the extent of the pulsations decreases in a rectilinear manner with an increase in temperature.

5. Between 12° and 27° C. the rate of pulsations increases in a non-rectilinear manner with an increase in temperature. With a further rise in temperature above 27° C. there is a rapid decrease in the rate of pulsations.

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INFLUENCE OF CYANIDE AND LACK OF OXYGEN ON THE ACTIVATION OF STARFISH EGGS BY ACID, HEAT AND HYPERTONIC SEA-WATER

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In previous papers (Lillie, 1915, 1917, 1926) I have called attention to the general resemblances between the phenomena of acid activation and of heat activation in unfertilized starfish eggs,—a parallelism indicating that both forms of activation are to be referred to the same kind of change in the egg system.[1] This primary or critical change in some way transforms the egg from the quiescent into the automatically developing or "activated" state. Since exposure to temperatures above the physiological range is known to produce acid (especially lactic acid) in many cells (as seen, *e.g.*, in the phenomena of heat rigor), the inference is natural that heat activation is in reality a secondary effect resulting from the production of acid within the egg; and the essential problem becomes that of determining the factors concerned in acid activation. Experiments on the relative activating effectiveness of a variety of weak penetrating acids having a wide range of dissociation constants (Lillie, 1926 and 1927) indicate that the essential factors in a given activating effect are (1) the production of a certain definite degree of acidity in some region of the egg (probably cortical) and (2) the maintenance of this acidity for a definite time. Some progressive change, the "activation process" or "activation reaction," occurs within the egg during this time; and if the egg is to be rendered capable of developing to an advanced stage, the process must advance to a certain stage of completion. One of the most striking features of the activation process in starfish eggs is that it is readily arrested at any stage by simply returning the eggs to normal sea-water; in such a case it may be renewed and brought to completion by a second exposure to heat or acid, applied after not too long an interval (Lillie, 1915, pp. 284 *seq.*). The rate of the activation process, in the case of a particular acid, appears closely proportional to the concentration of

[1] The chief parallels are (1) the similarity in the time-relations of partial and complete activation by the two methods; (2) the fact that both are completely effective only during the prematurational period of the egg; (3) the substitutability of one for the other in experiments on partial activation, and (4) other parallels described in the present paper.

the latter in the external medium—more specifically to the concentration of its undissociated molecules, which alone appear to penetrate freely to the site of the activation reaction (Lillie, 1926 and 1927).

Since apparently any non-toxic penetrating acid can activate starfish eggs, the special chemical nature of the acid would seem to be immaterial; its presence merely enables the activation process to proceed in the egg system. In general our present data indicate that the rate of activation is closely proportional to the acidity (cH above a certain critical level) attained at the site of activation. The most reasonable assumption appears to be that activation is an effect of the chemical union of certain specific substances already present or available in the egg,[2] and that this interaction, the primary or key reaction in the activating sequence, occurs only above a certain level of acidity (*i.e.*, within a certain pH range). The implication is that the product of this reaction—which we may call the "activating substance"—requires to accumulate to a certain definite level if complete activation is to result. Eggs in which this critical quantity of activating substance has been formed proceed normally with their development if placed under appropriate external conditions. That the accumulation of some material is a necessary condition of activation is indicated by the progressive character of the activation reaction, and especially by the possibility (already referred to) of arresting it at an incomplete stage and afterwards renewing it.

The activation reaction cannot be characterized definitely in chemical terms at present. The fact that responsiveness to activation, either by parthenogenetic agents or sperm, begins normally to decline at or about the time of separation of the first polar body and remains incomplete during the post-maturational period indicates a loss or destruction of some reactant or reactants at this time.[3] Completely mature eggs are capable of only partial activation: although such eggs still form fertilization-membranes and cleave after treatment with fatty acids or warm sea-water, only a small proportion (if any) develop to swimming stages; similarly only a minority of eggs fertilized with sperm during this period exhibit normal development. The capacity for complete activation is lost, apparently permanently, during the progress of the maturation divisions (Lillie, 1908).

[2] This is also the assumption of the fertilizin hypothesis of F. R. Lillie, which is quite consistent with the present view. *Cf.* Lillie, F. R.: "Problems of Fertilization," University of Chicago Press, 1919; also E. E. Just: "The Present Status of the Fertilizin Theory of Fertilization." *Protoplasma*, 1930, Vol. 10, p. 300.

[3] The term "prematurational period" is used in the present paper to designate the period between the dissolution of the germinal vesicle and the separation of the first polar body; the post-maturational period is that succeeding the separation of the second polar body.

This change in the physiological properties of the egg, like the change leading to its natural death if it is left unfertilized, (Loeb, 1902 *a* and *b*) is intimately dependent on normal respiration, as is shown experimentally by the effects of cyanide or deprivation of oxygen. If freshly removed starfish eggs are placed for two to four hours (at 20°) in sea-water freed of oxygen or containing KCN (M/500, M/1000), and are then returned to normal sea-water, they show an almost unimpaired response to fertilization or artificial activation; while the response of control eggs left for the same interval in normal sea-water is partial or defective. Such facts indicate that some material essential to the activation reaction (*i.e.*, some precursor or precursors) is removed or destroyed at a definite stage in the normal oxidative metabolism of the egg; apparently this occurs most rapidly during the period at which the polar bodies are being separated.

A further fact of significance is that the activation reaction, as such (*i.e.*, the reaction occurring during the actual exposure to fatty acid or heat), is independent of immediate oxygen consumption; that is, the eggs respond in a normal manner to heat or fatty acid while immersed in oxygen-free or cyanide-containing sea-water—even if they have previously been exposed for some hours to these media. Such suppression of oxidative metabolism has, however, a characteristic modifying influence on the rate of activation, as is shown by the experiments to be described below.

1. *Influence of Oxygen-lack or Cyanide on the Effect of a Second Activating Treatment Following Partial Activation by a First Treatment*

It was found in earlier experiments (Lillie, 1915, pp. 284 *seq.*) that two successive brief exposures to either heat or fatty acid, separated by a considerable interval (up to 30 minutes or more), may by a process of summation result in complete activation, although either exposure acting alone has only a partial effect.[4] A single continuous exposure lasting as long as the sum of the other two also causes complete activation. Such an experiment shows that the modification (whatever its nature) produced by the first exposure persists for some time, and that the second exposure induces further modification of the same kind. If the first exposure is made early, *e.g.*, within 5 to 10 minutes after the

[4] In partial activation the eggs form typical fertilization-membranes, but cleave slowly and irregularly (if at all) and die before reaching the blastula stage. In complete activation cleavage approaches the normal in rate and regularity and the great majority (in favorable cases all) of the eggs form active blastulæ and gastrulæ. All gradations in degree of activation can be obtained by varying the length of exposure.

commencing dissolution of the germinal vesicle, an hour or more may elapse without marked decline in the effect of the second exposure; later this exposure becomes progressively less and less effective. This decline in the response to a second activating treatment appears to follow the same course as the normal decline of activability already described. It also depends on the oxidative metabolism of the egg and can be greatly retarded by cyanide or removal of oxygen; apparently it is referable to a depletion of the reserve of activable material (precursor or precursors of the activating substance) left unchanged by the first treatment.

The following experiment (June 10, 1927) will illustrate. The mixed eggs from three starfish were exposed during the prematurational period for 6 minutes to .002 M acetic acid in balanced NaCl—$CaCl_2$ solution at 20° C. Part (*A*) was then transferred to normal sea-water (at *ca.* 18° C.) and part (*B*) to sea-water containing M/1000 KCN. At the intervals indicated in Table I eggs from both lots were again exposed for 6 minutes to the same acid solution and returned to normal sea-water. The total exposure, 12 minutes, is approximately the optimum for this solution at 20°. The intervals between the two successive exposures varied from 11 minutes to two hours. The results are shown in Table I.

TABLE I

Interval between exposures (*minutes*)	Percentage of eggs forming blastulæ as result of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs kept in M/1000 KCN in sea-water
11	90	90
20	60–65	90
30	60–70	90
45	60–70	70–80
60	60–70	80–90
90	50	70–80
120	5	50

Eggs exposed for 6 minutes without any second treatment showed typical partial activation, and a few (2–3 per cent) formed blastulæ. Sperm-fertilized eggs developed normally.

In normal sea-water nearly all the eggs have lost responsiveness to the second treatment after two hours, while of the eggs remaining in cyanide-containing sea-water for the same interval a large proportion developed. Table II describes a second similar experiment (July 11, 1927) with longer intervals between exposures.

TABLE II

July 11, 1927. The procedure was the same as in the previous experiment. The acid solution used was .0015 M acetic acid in Na–Ca solution at 20° C. A control series showed an optimum continuous exposure of 12–14 minutes, yielding 60–70 per cent blastulæ. The first exposure was 6 minutes; eggs so treated without a second exposure gave membranes and some cleavage but no blastulæ. After each interval the eggs kept in sea-water received second exposures of 6, 8, and 10 minutes; those kept in KCN sea-water received 4, 6, 8, and 10 minutes.

Interval between exposures (*hours*)	Percentage of eggs forming blastulæ and optimum durations of second exposure	
	A. Eggs kept in normal sea-water	B. Eggs in M/1000 KCN in sea-water
1	35–40 (8 and 10 min.)	*ca.* 50 (6–8 min.)
3	few (1%) (6–8 min.)	50–65 (4–6 min.)
5	0 (6–8 min.)	25–35 (4–8 min.)
22	(all dead by 22 hrs.)	0

Sperm-fertilized eggs developed normally.

Well-marked response to the second exposure after 5 hours in M/1000 KCN was observed in four other similar experiments and also in three experiments in which the eggs were left for 5 hours in oxygen-free sea-water between exposures. Four experiments with periods of 18 to 22 hours in M/1000 KCN showed almost no response after this interval, although in one experiment some slight effect was apparent after 19 hours.

Sea-water deprived of oxygen (by prolonged boiling, restoration of the original volume with boiled distilled water, and passage of a stream of hydrogen or nitrogen overnight) prolongs similarly the possible summation-interval. For example, on August 16, 1927, eggs kept for 2 1/2 and 5 hours in oxygen-free sea-water, after a first exposure of 5 minutes to .002 M acetic acid, yielded with both intervals 60–70 per cent of blastulæ as the result of a second exposure of 6 to 8 minutes to the same solution. The control eggs kept in normal sea-water showed almost no response to the second exposure. The eggs which received only the first exposure of 5 minutes showed typical partial activation with no blastulæ, while those receiving continuous exposures of 12, 13 and 14 minutes at the same time (30 minutes after removal from the animals) gave 75–90 per cent of blastulæ.

Seventeen experiments of this kind were performed during the summer of 1927 with results similar to those cited. In all cases the interval between the first inadequate and the second effective exposure was prolonged to several hours by the suppression of oxidative metabolism in the manner indicated.

These results are in conformity with the hypothesis that some material essential to the production of the activating substance disappears normally from the egg in the course of the regular oxidative metabolism. Hence the unused reserve of this material is retained within the egg for a longer period, together with the activating substance formed by the first exposure, if oxygen-consumption is prevented.

There is, however, some indication that the reserve of precursor material (activable substance) remaining in the egg after the first exposure is not retained entirely unaltered during the interval, but that it undergoes gradual transformation into activating substance. This is seen in the fact that partially activated eggs show an increase in the degree of their activation (as compared with eggs of the same lot left in normal sea-water), if they are kept undisturbed for some hours in oxygen-free or cyanide-containing sea-water. For example, in the experiment just cited (August 16, 1927) eggs left for two hours in oxygen-free sea-water (after the initial exposure of 5 minutes to .002 M acetic acid), and then transferred to normal sea-water, yielded without any second treatment 10–15 per cent of blastulæ, while after remaining for 5 hours in oxygen-free sea-water more than 50 per cent formed blastulæ. In the latter case (5 hours without oxygen) a second exposure of two minutes proved almost as effective as one of 6 minutes, both yielding 65–70 per cent of blastulæ. Such cases show that a partial activation may be completed in many eggs by simple exposure of one to several hours to cyanide-containing sea-water. This effect is similar to Loeb's completion of activation in sea-urchin eggs, after membrane-formation by acid, by after-treatment with cyanide-containing sea-water (Loeb, 1913). In starfish eggs a well-marked shortening of the optimal duration of the second acid treatment was a constant effect of one or more hours exposure to oxygen-free or cyanide-containing sea-water. Apparently prolonged suppression of oxygen-consumption in the partially activated eggs has a physiological effect similar to that of treatment with acid or high temperature.

It seems probable that the production of acid within the egg under the conditions of anaërobic metabolism is responsible for the increase of activation shown in such cases. This supposition is consistent with the fact that previous exposure of normal untreated starfish eggs to oxygen-free or cyanide-containing sea-water also very definitely increases the susceptibility to activation by either heat or acid, as will be shown in the next section. I have, however, never succeeded in activating such eggs, even to the extent of membrane-formation, by this treatment alone.[5] The acid formed under these conditions may

[5] E. P. Lyon obtained parthenogenetic development in the *Strongylocentrotus* egg at Naples by prolonged exposure to solutions of KCN (M/100 to M/1000) in sea-water (*cf.*, *Am. Jour. Physiol.*, 1903, Vol. 9, p. 308).

not reach the critical level of concentration required for activation, or it may be ineffective for some independent reason.

2. *Activation after Previous Exposure of Normal Eggs to Cyanide and Lack of Oxygen*

In the experiments just described partially activated eggs after remaining some hours in oxygen-free or cyanide-containing sea-water showed a tendency to complete their activation spontaneously; *i.e.*, the second effective exposure to acid was unexpectedly brief, and many eggs formed blastulæ without any second exposure. Similarly, normal unactivated eggs kept under the same oxidation-suppressing conditions show in course of time an increasing susceptibility to activation by either heat or acid. Suppression of oxygen-consumption has thus a double effect on the eggs: (1) prolongation of the time during which they remained activable, and (2) increase of activability, as shown by a decrease in the effective durations of exposure to the activating agent.

Cyanide-containing Sea-water.—A typical series in which the eggs were exposed to KCN-containing sea-water for 2 1/2 hours before the activating treatment is the following:

July 3, 1929. Eggs from several starfish were placed, 35 minutes after removal, in sea-water containing M/1000 KCN. Two hours, 35 minutes later, a part (*B*) was washed in several changes of normal sea-water, left in sea-water for 26 minutes and then placed in M/260 butyric acid solution in sea-water. A second part (*C*) after 2 hours, 32 minutes in the KCN sea-water was transferred directly to KCN sea-water containing also M/260 butyric acid.[6] The essential difference between *B* and *C* is that in *B* the activating treatment occurred after the eggs were washed free of cyanide, while in *C* the entire period preceding and during activation was spent in the presence of cyanide. At two-minute intervals eggs were transferred from the butyric acid

[6] In estimating the rate of activation in solutions of fatty acid containing KCN, allowance must be made for the neutralizing action of this salt, which is very nearly the same as that of $NaHCO_3$. In general the rate of activation exhibited by starfish eggs placed directly from sea-water in solutions of fatty acid containing also KCN is closely similar to that of eggs exposed to solutions of the same non-neutralized acid strength without KCN. This was shown experimentally as follows: Eggs exposed to balanced NaCl-$CaCl_2$ solution containing both acetic acid and KCN in equal concentrations, .002 M, showed no membrane formation or other signs of activation with exposures from 5 to 18 minutes. The control experiment with .002 M acetic acid alone showed optimal activation at 10 minutes, with 75–80 per cent of eggs forming blastulæ. The same NaCl-$CaCl_2$ solution containing .002 M acetic acid plus .001 M KCN gave a rate of activation similar to that of .001 M acetic acid without KCN. The solution used above, sea-water containing M/260 butyric acid *plus* M/1000 KCN, corresponds closely in its activating effect on normal eggs to a solution of .003 M butyric acid in sea-water (*i.e.*, M/260, *ca.* .0038–.001 M).

solutions to normal sea-water; later the proportions developing to blastulæ were determined. The control (*A*) consisted of eggs exposed to M/260 butyric acid in sea-water, beginning 40 minutes after removal from the animals.

Table III gives the approximate percentages of eggs forming blastulæ with the different exposures.

TABLE III

Duration of exposure (20° C.) (*minutes*)	Percentage of eggs forming blastulæ		
	A. (Control without KCN exposed to normal sea-water containing M/260 butyric acid)	B. (KCN 2½ hrs. exposed to normal sea-water containing M/260 butyric acid)	C. (KCN 2½ hrs. exposed to sea-water containing M/260 butyric acid *plus* M/1000 KCN)
2	0	40–50	no observation
4	1	30–40	60–70
6	15–20	30–40	60–70
8	30–40	60–70	50
10	*ca.* 90	70–80	20–30
12	80–90	70–80	*ca.* 10
14	70–80	30–35	*ca.* 10
16	20–30	*ca.* 10	1

Nearly half the eggs in Part *B* formed blastulæ with exposures as brief as two minutes; also a large proportion continued to develop favorably with longer exposures up to 12 minutes. The effect is even more striking in Part *C*. Here the effective (non-neutralized) concentration of acid in normal sea-water (*ca.* .003 M) typically requires 20 minutes or more for complete activation; yet after the preliminary treatment with KCN, two-thirds of the eggs formed blastulæ with exposures of 4 to 6 minutes.

Two other series on July 15 and 16, with preliminary exposures of 3 and 5 hours (respectively) to KCN sea-water gave closely similar results. In the series of July 15 eggs exposed to M/260 butyric acid in sea-water, after 3 hours in KCN sea-water, for periods of one to 6 minutes, gave 60–80 per cent blastulæ. With the cyanide-containing butyric solution one minute was ineffective, but exposures of 2 to 6 minutes yielded 50 per cent or more blastulæ. The results of July 16, with 5 hours previous treatment with KCN, were similar; butyric acid in normal sea-water showed optimal activation at 2 minutes with 70–80 per cent blastulæ; the same solution in KCN-containing sea-water was ineffective at 2 minutes, but with exposures of 4 to 14 minutes gave 50 per cent or more blastulæ. On July 17 and 18 similar series with 5

and 3 hours in KCN-sea-water showed the same effect with somewhat fewer blastulæ.

The shortening of the effective exposures under the influence of KCN is appreciable although slight after 15 or 20 minutes and increases with time. Table IV gives the results of a typical series in which the preliminary exposure to KCN was varied from 15 to 65 minutes. The optimum exposure for the control eggs unexposed to KCN was 10 minutes (21° C.), with more than 90 per cent forming blastulæ.

TABLE IV

Experiment of June 20, 1930. Eggs were exposed during the prematurational period to M/260 butyric acid in normal sea-water at 21° C. after washing free of cyanide.

Previous Exposure to M/1000 KCN (*minutes*)	Times of Exposure to Butyric Acid (*minutes*) and Percentages of Eggs Forming Blastulæ								
	2	3	4	5	6	7	8	9	10
None (control)	0	0	0	2–3	2–3	5–10	*ca.* 50	65–75	90
15	0	0	0	2–3	2–3	60–70	60–70	75–85	80–90
26	0	*ca.* 1	*ca.* 1	*ca.* 1	25–35	50–60	70–80	70–80	*ca.* 50
45	1	*ca.* 5	10–20	40–50	60–70	65–75	50–60		
65	*ca.* 1	*ca.* 5	10–15	20–25	35–45	55–65	25–35		

Three other similar series carried out between June 17 and June 23, 1930, with exposures to KCN ranging from 12 minutes to 5 hours, gave the same general result. In general, the degree of shortening increases with duration of exposure up to a maximum of 2 to 3 hours. After 5 hours there is usually a decline of responsiveness, and after 20 to 24 hours no blastulæ were obtained, although there was some partial activation.

Oxygen-free sea-water.—Experiments with eggs remaining for 2 to 5 hours in oxygen-free sea-water before activation with fatty acid gave a similar result.[7] Eight successive series between July 23 and August 3, 1929 showed uniformly a striking decrease in the activating exposures; of such eggs a large proportion and in some cases nearly all formed blastulæ after exposures of one to 2 minutes to M/260 butyric acid. Table V gives a summary of the first four series. The eggs, after exposure to the oxygen-free sea-water (in flasks through which a slow stream of hydrogen was kept flowing) for the periods named, were treated for

[7] In general the physiological effects of exposing starfish eggs to cyanide-containing and to oxygen-free sea-water are strikingly similar. One constant effect following exposure for one to several hours to either medium is to render the eggs glutinous or sticky, so that they cohere in masses, a result (apparently) of the secretion of some water-swelling or glutinous material.

periods ranging from one to 16 minutes with M/260 butyric acid made up in both normal and oxygen-free sea-water. The controls, as before, consisted of eggs exposed during the prematurational period (usually 30 to 45 minutes after removal) to M/260 butyric acid,[8] again both with and without oxygen. These showed optima varying between 6 and 12 minutes (most frequently at 8 minutes) with 70 to 85 per cent of eggs forming blastulæ. The temperatures of the sea-water and butyric acid solutions were 21° to 22.5° C.

TABLE V

Experiment and date (1929)	Control (Effective exposures and percentages of eggs forming blastulæ)		Period in O_2-free sea-water before activation		Exposures to M/260 butyric acid and percentages of resulting blastulæ			
					(a) O_2-containing acid solution		(b) O_2-free acid solution	
	min.	%	*hrs.*	*min.*	*min.*	%	*min.*	%
1. July 23	6	(35–40)	2	15	2	(*ca.* 50)	2	(*ca.* 50)
	8	(70–80)			4	(35–45)	4	(50–60)
	10	(50–60)			6, 8	(25–35)	6	(35–40)
					10	(20–35)	8, 10	(25–35)
							12	(20–25)
2. July 24	4	(25–35)	5		1	(40–50)	1	(35–40)
	6	(80–90)			2, 3	(20–30)	2	(30–35)
	8	(50)			4	(5–10)	3	(15–20)
	10	(5)			6	(0)	4	(*ca.* 1)
3. July 25	6	(40–50)	4	25	1, 2	(80–90)	1	(75–85)
	8	(75–80)			3, 4	(75–85)	2, 3	(80–90)
	10	(*ca.* 90)			5	(70–80)	4, 5	(70–80)
	12	(50–60)			6, 7	(*ca.* 60)	6, 7	(65–75)
					8	(50)	8	(55–60)
4. July 26	8	(10–15)	3	40	2, 4	(70–80)	2, 4	(70–80)
	10	(40–50)			6, 8	(60–70)	6	(60–65)
	12, 14	(70–80)			10	(*ca.* 50)	8	(*ca.* 50)
					12, 14	(25–35)	10	(30–40)
							12	(*ca.* 5)

In three other series (August 1, 2 and 3, 1929) eggs kept previously for equal times in both KCN-containing and oxygen-free sea-water (respectively 2 1/4, 3 and 4 1/2 hours) were exposed to M/260 butyric acid in sea-water. In all cases exposures of 1, 2 and 3 minutes gave numerous blastulæ (60 to 80 per cent in five of the six experiments); exposures of 4, 5 and 6 minutes were less effective, and with 8 minutes

[8] Two cc. M/10 butyric acid *plus* 50 cc. sea-water. Oxygen-free sea-water was used in mixing solution (*b*) and hydrogen was bubbled through the mixture for some hours.

almost no blastulæ were formed. The control eggs gave no blastulæ with the two-minute exposure, few with 4 minutes and a maximum (60–80 per cent) with 6 to 8 minutes.

One uniform feature of these experiments was that the eggs, after prolonged suppression of oxidative metabolism, not only gave complete activation with brief exposures to acid, but also showed a less sharply defined optimal period of exposure; *e.g.*, in the experiments of July 25th and 26th exposures ranging from one to 8 minutes were almost equally effective in producing blastulæ (65 to 85 per cent). This behavior differs strikingly from that of eggs activated in normal sea-water soon after removal; in this case the curve relating percentages of blastulæ to durations of exposure rises rapidly to a maximum—usually at 8 to 10 minutes at this temperature (21°–22° C.) and concentration of acid—and declines equally rapidly, appearing nearly symmetrical.[9] Under the anaërobic conditions just described, over-exposure seems less deleterious; the optimum is reached early, and with further prolongation of the activating treatment there is a more gradual decline of effect; *i.e.*, over-activation is caused less readily, an indication (possibly) of a partial depletion of activable or precursor material during the interval in oxygen-free sea-water. A similar prolongation of the optimum is seen in eggs remaining for some hours in KCN-sea-water previous to activation.

Controls of sperm-fertilized eggs were also kept during these experiments: at the end of the period in oxygen-free or KCN-containing sea-water eggs were transferred to normal sea-water, washed by changing the latter, and immediately fertilized by sperm. In general, responsiveness to sperm fertilization was found to run closely parallel with activability by artificial agents; *i.e.*, normal fertilizability is also prolonged for several hours by exposure to anaërobic conditions. Typically, sperm-fertilization is complete and uniform only during the prematurational period and falls off rapidly with the separation of the polar bodies (Lillie, 1908). Both kinds of prolongation of responsiveness are to be regarded as expressions of the same kind of change in the egg; in terms of the foregoing hypothesis, the precursor material, which is transformed into activating substance under the influence of both acid and sperm, is retarded in its breakdown or removal by the suppression of oxidative metabolism.

3. *Influence of O_2-lack and KCN on Activation by Heat*

The effective exposures to activating temperatures (*e.g.*, 32°) are similarly shortened by previous exposure to cyanide or lack of oxygen.

[9] *Cf.* the curves in my article on activation by acids in *Jour. Gen. Physiol.*, 1926, Vol. 8, p. 339.

In general, however, I have found that in the case of heat activation the decline in responsiveness during such exposure is more rapid than with acid activation. This may indicate that the material which gives rise to the activating acid (presumably carbohydrate) undergoes comparatively rapid depletion with time. In some cases, the eggs have preserved good responsiveness to heat for as long as 3 hours in KCN-containing sea-water, although usually the response is greatly diminished by this time. Apart from this difference the general phenomena are closely similar.

The procedure was the same as in my earlier experiments on heat activation (Lillie, 1908, 1915). The eggs, together with a small quantity of sea-water, were placed in a beaker (or small flask) to which 100–200 cc. of sea-water at the required temperature (32°) was added. The temperature was kept constant by a water bath. At intervals eggs were transferred to a series of dishes containing normal sea-water.

The following experiment (August 6, 1929) is cited as an illustration. One portion of starfish eggs (*A*) was placed, one half hour after removal from the animals, in M/1000 KCN in sea-water; another portion (*B*) in oxygen-free sea-water; in this case one to 2 cc. of a dense egg suspension was added to 100 cc. of oxygen-free sea-water contained in a flask through which hydrogen had flowed overnight, and a slow flow of H_2 was continued. After 3 hours eggs from both lots were treated with normal sea-water at 32° for the times indicated. It had previously been found that eggs left in normal sea-water for 2–3 hours, and then warmed, show only partial response and never form blastulæ (Lillie, 1908).

TABLE VI

Treatment	Percentage of eggs forming blastulæ after exposure to 32° C. for the times indicated (*minutes*)							
	1	2	3	4	5	6	7	8
1. Control: warm sea-water, 30 min. after removal.....	0	1	2–4	25–35	75–85	75–85	60–70	25–35
2. (*A*) In KCN-sea-water 3 hrs., 10 min. before warming.....	10–15	25–35	*ca.* 50	25–35	20–30	1–2	0	0
3. (*B*) In O_2-free sea-water 3 hrs., 10 min. before warming.....	*ca.* 1	*ca.* 5	20–25	30–35	5–10	1–2	0	0

The preservation of responsiveness and the shortening of the effective exposures are both well shown in this series. In six other similar

experiments during the same season, with exposures of one hour or more to KCN, the effective exposures were similarly decreased, but fewer eggs formed blastulæ. With a briefer previous stay in KCN-containing-sea-water (19 to 24 minutes in five experiments between June 25 and July 1, 1930), the decrease was less marked but still definite. The actual exposure to heat may be made either in normal or in KCN-containing sea-water with the same result. Even without a previous stay in KCN-containing-sea-water, eggs warmed in KCN-containing or oxygen-free sea-water show a distinctly briefer optimal exposure than eggs of the same lot warmed in normal sea-water. Table VII gives a summary of experiments illustrating these effects.

TABLE VII

Procedure	Percentages of eggs forming blastulæ after exposure to 32° for the times indicated (*minutes*)								
	1	2	3	4	5	6	7	8	9
1. June 25, 1930. (*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (few membranes)	0 (all form membranes)	0	10–15	10–15	20–25	30–35	*ca.* 10
(*b*) No previous exposure to KCN; KCN-containing sea-water at 32° at same time as control	0 (membranes in nearly all eggs	0	0	10–20	20–30	35–40	5–10	0	
2. June 28, 1930. (*a*) Control: normal sea-water at 32°	0 (no membranes)	0 (50% membranes)	0 (all membranes)	*ca.* 1	25–35	60–65	*ca.* 60	10–20	
(*b*) Previous exposure to KCN sea-water for 17 minutes; then direct to KCN-sea-water at 32°	0 (all form membranes)	0	5–10	15–25	60–70	20–30	1	0	

Lack of oxygen has the same effect as KCN upon the rate of heat activation. One uniform and readily observed effect is the shortening of the exposures required for membrane-formation. Typically eggs exposed to normal sea-water at 32° for one minute do not form mem-

branes or show any other signs of activation; exposures of 2 1/2 to 3 minutes are required to form membranes in a majority of eggs. But if eggs are warmed in KCN-containing or O_2-free sea-water (especially after previous exposure to either medium) for one minute, all (or nearly all) form membranes (*cf.* Table VII). Restoration of normal oxygen-consumption reverses this sensitivity,—*i.e.*, eggs exposed as above to oxygen-free or cyanide-containing sea-water and then returned for a few minutes to normal sea-water are found to require 2 or 3 minutes at 32° for membrane-formation, like previously untreated eggs.

Membrane-formation is an index of partial activation and is a regular effect of brief exposure to either heat or acid, as well as to other parthenogenetic agents such as solutions of cytolytic compounds and pure isotonic alkali salt solutions. Complete activation, however, is obtained only with heat and acid,[10] but with these agents the exposures required for the formation of blastulæ are several times longer than those required for membrane-formation alone. In confirmation of the hypothesis that heat acts indirectly through the intra-cellular production of acid, the following experiment is cited. Eggs were exposed to neutral, acid and alkaline artificial unbuffered sea-water (van't Hoff's solution) [11] at 34° as indicated in Table VIII.

TABLE VIII

Solution in which eggs were warmed (34°)	Times of exposure (*minutes*) and percentages of resulting blastulæ								
	1	1½	2	2½	3	3½	4	4½	5
1. Neutral van't Hoff	0 (*ca.* 50% membranes)	2–3	20–30	65–75	50–60	*ca.* 50	15–20	10–15	1
2. van't Hoff *plus* N/1000 NaOH	0 (40–50% membranes)	*ca.* 5	30–40	*ca.* 90	*ca.* 90	75–85	20–30	1–2	0
3. van't Hoff *plus* N/1000 HCl	25–30 (*ca.* 100% membranes)	55–65	75–85	20–30	1	0	0	0	0

Neutral or moderately alkaline balanced isotonic salt solution at 34° has the same action as sea-water at this temperature, while acidulation to this degree nearly doubles the rate of activation. This is

[10] *I.e.*, apart from hypertonic sea-water (*cf.* next section), which apparently acts indirectly through its influence on metabolism. In numerous experiments with cytolytic compounds (*e.g.*, ether and chloroform), pure Na-salt solutions, ultra-violet radiation and the electric current, I have never obtained more than partial activation, whatever the duration of exposure.

[11] Made by mixing M/2 solutions of the following salts in the proportions by volume: 100 NaCl, 7.8 $MgCl_2$, 3.8 $MgSO_4$, 2.2 KCl, 2.0 $CaCl_2$.

apparently a summation effect resulting from a partial penetration of the external acid to the site of activation. The increased rate of activation observed in KCN-sea-water may similarly be regarded as a summation effect, due to the increased intracellular formation of acid under the conditions of asphyxiation. With regard to the reversal of hypersensitivity just described, it may be assumed that readmission of oxygen restores the egg to its original condition through the oxidative removal of the surplus of acid, as in the case of the lactic acid formed in muscle cells under asphyxia.

Experiments in which the eggs were placed for 19–20 minutes in sea-water saturated with pure oxygen from an oxygen-cylinder and then warmed to 32° in the same medium showed no significant difference from the control. (Results of three series: July 15, 16 and 18, 1930.) Apparently an increase of oxygen tension to 5 times the normal does not affect the rate of heat activation.

4. *Influence of O_2-lack and KCN on Activation by Hypertonic Sea-Water*

The case of hypertonic sea-water is peculiar. This agent, acting alone, is not so effective with starfish eggs as heat or fatty acid, and its rate of action is much slower. Nor is it so effective with these eggs as with *Arbacia* eggs. It is unusual to obtain 50 per cent of free-swimming blastulæ with hypertonic sea-water alone, although in a few experiments the proportion has reached 70 per cent or even higher. The most favorable procedure is to expose the eggs during the pre-maturational period to a mixture of 100 volumes sea-water *plus* 20 volumes 2.5 M NaCl for 1 1/2 to 2 hours (at 20–22°). Exposures during the post-maturational period are much less effective. In experiments with a series of hypertonic mixtures, consisting of 100 volumes sea-water *plus* (respectively) 10, 15, 20, 25 and 30 volumes 2.5 M NaCl, membrane-formation and blastulæ were obtained with all but the first and last. The effective osmotic gradient thus has a well-defined range; and within this range the optimal exposures show in general a decreasing duration with increasing concentration, as in Loeb's experiments with *Strongylocentrotus*.[12] The best results have been obtained with the 100 + 15 and 100 + 20 mixtures, corresponding to an increase in osmolar concentration of 50 to 60 per cent. The second of these solutions was used in most experiments.

Besides acting as a single parthenogenetic agent, hypertonic sea-water may also be used to supplement and complete a partial activation by heat or fatty acid (See Lillie, 1915, pp. 284 *seq.*).

A characteristic peculiarity in the mode of action of this agent is

[12] J. Loeb: Artificial Parthenogenesis and Fertilization, Chapter XI.

that the physiological change which it produces in the egg during the period of exposure is closely associated with the consumption of oxygen. In this respect the action of hypertonic sea-water offers a contrast to that of fatty acid and heat, both of which agents act independently of immediate oxygen consumption, besides being more uniform, rapid and complete in their activating effect.

Loeb showed many years ago that oxygen-free or KCN-containing hypertonic sea-water was ineffective as an after-treatment in sea-urchin eggs which had received the initial membrane-forming treatment.[13] In starfish eggs similar conditions are found, except that in order to deprive the hypertonic sea-water completely of its activating influence, it is necessary to expose the eggs previously for some time to cyanide or lack of oxygen. Eggs transferred from normal sea-water directly to oxygen-free or KCN-containing hypertonic sea-water always show some partial activation (*i.e.*, membrane-formation and occasionally a few blastulæ); while if they are kept first in oxygen-free (or KCN-containing) isotonic sea-water for 2 to 3 hours and then exposed to the oxygen-free (or KCN) hypertonic sea-water for the usual time (1 1/2 to 2 hours) the great majority show no signs of activation. If such eggs are then returned to sea-water and fertilized with sperm, a large proportion develop to larval stages, showing that they have remained essentially unchanged in their properties.

These conditions are illustrated by the experiments described in Tables IX and X.

TABLE IX

June 26, 1929. The eggs were placed, 40 minutes after removal, in (*A*) hypertonic sea-water of the composition 100 volumes sea-water *plus* 20 volumes 2.5M NaCl, and (*B*) in the same solution *plus* M/1000 KCN. Temperature 21° C. At intervals of 1½, 1¾ and 2 hours portions were transferred to normal sea-water.

Duration of exposure (*hours*)	Percentage of mature eggs forming blastulæ	
	A. Hypertonic sea-water	B. Hypertonic sea-water *plus* M/1000 KCN
1½	70–80	0
1¾	60–70	1–2
2	60–70	*ca.* 5

The eggs in this experiment were exceptionally responsive to hypertonic sea-water; it is in fact unusual to obtain any blastulæ after treatment with KCN-containing hypertonic sea-water. In a similar experiment on June 27 the eggs exposed to cyanide-free hypertonic sea-water

[13] *Loc. cit.*, Chapter XI.

for 1 1/2 hours at 23° formed 25 per cent of blastulæ, while with KCN present, although all formed membranes and many cleaved irregularly or fragmented, no blastulæ were obtained. Other experiments gave similar results.

The effect of placing in oxygen-free or KCN-containing sea-water for some time previous to the hypertonic treatment is shown in the following experiments (August 1, 1929).

TABLE X

Procedure	Result
A. Eggs exposed to hypertonic sea-water (100 + 20) alone, beginning 30 min. after removal, for 1½ and 1¾ hours (23°)	Activation in all; 25–35 per cent blastulæ
B. Eggs in O_2-free sea-water for 2 hours, beginning 30 min. after removal; thence to O_2-free hypertonic sea-water for 1½ and 1¾ hours	Great majority show no activation (no membranes); partial activation in a few
C. Eggs in sea-water + M/1000 KCN for 2 hours, beginning 35 minutes after removal; thence to KCN-containing (M/1000) hypertonic sea-water for 1½ and 1¾ hours	Similar to Experiment B. No activation except in small minority

A similar result was obtained in eight other experiments of the same kind, five with KCN-containing and three with oxygen-free hypertonic sea-water. It is evident that when the oxidative metabolism of the egg is suppressed, the abstraction of water by hypertonic media has little or no activating effect. The period of asphyxiation does not in itself deprive the eggs of responsiveness to hypertonic treatment, provided the oxygen is later restored. Eggs returned after 2 to 3 hours in KCN-containing sea-water to normal sea-water, washed thoroughly in sea-water and then exposed to hypertonic treatment show typical activation with production of blastulæ. As already shown, such eggs also retain their responsiveness to activation by heat and acid and to sperm-fertilization.

THEORETICAL

Assuming that the activation process consists in a regular sequence of interconnected physical and chemical processes, leading to some definite modification of the egg-system, we have to inquire how such a sequence can be initiated by a temporary slight rise of acidity. In general the experimental facts suggest that the first step in the chemical

sequence of activation is a transformation (*e.g.*, hydrolysis) of some compound present in the surface layer or cortical zone of the egg, this change leading directly or indirectly to the formation of some specific metabolic product ("activating substance"), the accumulation of which renders the egg capable of automatic development. Just how such a substance may be regarded as producing its effect is uncertain; it might contribute to special cell-structure, or alter already existing structure (*e.g.*, increase permeability), or it might be a catalyzer, or form some necessary link in the chain of structure-forming reactions. On the whole the last supposition seems the most likely one. Examples of the control of morphogenetic processes by special metabolic products are well known (*cf.*, the case of hormones); and they show at least that the hypothesis of an activating substance is consistent with the general facts of developmental physiology.

It seems unlikely that the primary or releasing reaction (or reactions) of activation can be widely different from those met with in other types of reactive cell. In general the basic chemical processes of protoplasm (as well as its basic chemical composition) show a well-defined uniformity throughout the range of living organisms. Thus we find that the carbohydrate metabolism of yeast cells resembles closely that of muscle cells, with certain divergences in detail; the utilization of oxygen by living cells depends on factors of a kind universally present in protoplasm (*cf.*, the heme and sulphydryl compounds); the course and end-products of protein metabolism are similar in their general character. Such considerations lead us to expect that the key reactions of activation may be simple and of a type already known.

The prompt arrest of the activation reaction when eggs are returned from acid to normal sea-water, and its equally prompt renewal on re-exposure to acid, are facts suggesting that the rise of acidity within the egg acts by releasing from combination some compound which is necessary for the specific reaction of activation, and that this releasing reaction is reversed on return to or toward neutrality, the compound re-entering combination and becoming again unavailable. The releasing reaction itself has apparently the character of a hydrolysis, and the dependent specific reaction which forms the activating substance is to be regarded as keeping pace with this hydrolysis.

Certain readily hydrolyzable compounds widely distributed in cells show a behavior of the general kind required by such a hypothesis. Of chief interest are the phosphate compounds or esters, especially the guanidin phosphates (phosphagens), which appear to be of special importance in the metabolic reactions associated with excitation in

muscle. The enzymatic hydrolysis of these compounds in muscle extracts is influenced by variations of acidity in a manner closely paralleling the characteristic variation in the rate of activation of eggs with concentration of acid. The creatin phosphate of vertebrate muscle extracts splits into creatin and phosphoric acid at a slightly acid reaction (pH = 6.4), and is resynthesized at slight alkalinity (pH = 8.5–9); the analogous arginin phosphate of invertebrates shows similar properties, but has a greater tendency to resynthesis at slightly alkaline or even neutral reaction.[14] The total phosphate content of starfish eggs is very similar to that of muscle cells (Page, 1927), and it is possible that in the activation and other reactions of these cells phosphate esters play a rôle of the same kind as in the analogous reactions of muscle and presumably other cells. Meyerhof has suggested that a special biological rôle of the guanidin compounds may consist in the binding and splitting off of phosphate groups (Meyerhof and Lohmann, 1928), a view in accordance with the general importance of phosphates in cell metabolism. The hypothesis that the splitting of phosphagen or some analogous compound is the primary chemical event in activation requires, however, further experimental testing from both the physiological and the biochemical sides.[15]

With regard to the activation by hypertonic sea-water, it is to be noted that abstraction of water from the egg means increase in concentration of egg-constituents, and it is possible that locally (*e.g.*, in the cortical region) this increase may be considerable. Conditions favorable to the synthesis of complex compounds would thus arise. The general theory of the dehydrolytic synthesis of complex molecules through the combination of a number of smaller molecules has recently been reviewed by Wasteneys and Borsook (1930, 1931) in relation to their work on the enzymatic synthesis of plasteins from the products of peptic hydrolysis. The authors show both theoretically and experimentally that increase in the concentration of such a digest may change the prevailing direction of the reversible reaction from a hydrolysis to a synthesis; and it is reasonable to assume that conditions of the same general kind exist in living cells. In the case of the starfish egg the inference would be that hypertonic sea-water acts by promoting the synthesis of some compound (*e.g.*, protein) which either is, or gives rise to, the activating substance.

[14] Meyerhof and Lohmann, *Biochem. Zeitschr.*, 196, 49, 1928. It might be objected that such reactions are too slow to account for the rapid course of the activation reaction in starfish eggs immersed in acid sea-water, and its rapid arrest on return to normal sea-water; but it is well-known that many enzymatic reactions which are slow *in vitro* proceed rapidly in the living cell.

[15] *E.g.*, it would be important to determine if the free phosphate is increased in the egg during activation, as in the stimulation of muscle and nerve.

It is significant that the formation of the activating substance appears to be promoted by two quite different external changes of condition, hypertonicity and moderate acidity. As already described, hypertonic activation proceeds relatively slowly as compared with acid activation and is dependent on oxygen consumption. The essential problem is why dehydration combined with oxidation should lead to the production of the same substance as slight local increase of acidity. Experimentally we find that the two parthenogenetic procedures may act additively or supplement each other; according to the present hypothesis, however, they influence metabolism in different ways, the one promoting synthesis of a complex compound, the other releasing hydrolytically some relatively simple compound which enters further combination. The fact that both procedures have the same final physiological effect suggests that the activating substance is formed by the combination of the two products. In such a case, according to the mass action rule, increase in the concentration of the one product would offset deficiency in the other; *i.e.*, activation would result from a sufficient increase in either compound, provided some of the other were available. This would explain why either acid or hypertonic sea-water acting alone may cause complete activation, since both compounds may be assumed to be present in low concentration in the resting egg. Whether the additive relations between the two parthenogenetic procedures conform to such a hypothesis in detail can be determined only by further experiment.

Summary

1. Suppression of oxygen-consumption in freshly removed unfertilized starfish eggs (by exposure to cyanide-containing or oxygen-free sea-water) prolongs by several hours the period during which they remain responsive to artificial activation by heat, acid, or hypertonic sea-water (as well as to sperm fertilization). The possible interval between a first partial and a second completing activation may be similarly prolonged.

2. During the exposure of the eggs to these asphyxiating conditions their susceptibility to activation by heat or acid, as indicated by the effective durations of exposure, undergoes a progressive increase.

3. Eggs kept for some hours in cyanide-containing or oxygen-free sea-water and then exposed to acid or heat while immersed in these media show normal activation. On the other hand, a similar suppression of oxygen consumption prevents activation by hypertonic sea-water.

4. It is suggested that the activation by hypertonic sea-water de-

pends on the increased intracellular production, by dehydrolytic synthesis, of some complex specific compound (*e.g.*, protein); while in the anaërobic activation by acid (or heat) the critical change is a hydrolysis (*e.g.*, of a phosphagen compound), yielding a product which combines with the complex compound to form the specific activating substance. The accumulation of this substance to a certain definite level determines activation. Two metabolic processes, respectively aërobic and anaërobic, would thus coöperate in activation. The fact that either hypertonic sea-water or acid (or heat), acting alone, can produce the same physiological end-effect, complete activation, is shown to be consistent with this hypothesis.

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MODIFICATION OF THE RATE OF OXYGEN CONSUMPTION BY CHANGES IN OXYGEN CONCENTRATION IN SOLUTIONS OF DIFFERENT OSMOTIC PRESSURE

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INTRODUCTION

It has not been possible to derive a general rule describing the relation between rate of oxidations in organisms and the oxygen tension of the environment. Pflüger (1872), reasoning from observations on the oxygen-absorbing power of the blood and the partial pressure of oxygen in the alveoli of the lungs in the dog, concluded that the rate of oxygen absorption of the tissues is independent of the oxygen tension over a wide range. Thunberg (1905), testing the oxygen absorption of *Lumbricus*, *Limax*, and *Tenebrio* larvæ in different oxygen tensions, found that the rate varies with the oxygen tension. Thunberg assumed that the oxygen tension within the cells is less than that of the milieu, but not zero, and concluded that his results were in accord with the fact that the velocity of a reaction depends on the number of molecules reacting; hence the velocity of oxidations in the organism varies with the oxygen concentration of the environment until a maximum is reached. Henze (1910), from his experiments with pelagic forms, concluded that in organisms with thin and delicate tissues the oxygen tension of the cells is that of the surrounding water and always in excess of requirements; consequently in such organisms the rate of oxygen utilization is independent of the oxygen tension of the water.

Krogh (1916) amplified Henze's conclusion and states that whether or not the rate of oxygen consumption is independent of the oxygen concentration of the environment depends on two conditions: In animals with well developed blood streams and respiratory systems, and in animals with a low percentage of dry substance, the oxygen tension does not affect the rate of oxygen utilization over a wide range. The assumption is necessary in these cases that the oxygen tension in the tissues is positive. On the other hand, if the organism has a high proportion of dry substance and "imperfect" respiratory and circulatory systems, the rate of oxygen utilization is highly

dependent on the oxygen tension of the environment until the oxygen tension is above the normal. It is assumed that the oxygen tension of the tissues of such forms approaches zero.

Hyman (1929) suggests that, in certain cases at least, the body surface acts as a regulatory mechanism; this appears to act according to internal requirements and hence in the presence of excess oxygen the oxidative rate remains constant. Shoup (1929) holds that the size of the organism is important. In general, according to Shoup, microscopic forms are small enough to allow complete diffusion of the dissolved gases. In other words, they are independent of the oxygen concentration, within limits, because oxygen is always present in excess within the system. Shoup also adopts the view expressed by Krogh (1916) that in large organisms the rate of respiratory exchange depends on oxygen tension and the adequacy of the respiratory and circulatory systems.

Thus far it is not possible to explain the experimental evidence at hand by any or all of these theories. According to Krogh, the water content of the organism is an important characteristic in determining its response to changes in the oxygen tension of the environment. Thus an organism with relatively low water content and what Krogh calls "imperfect" systems of respiration and circulation might be expected to consume oxygen in direct dependence on the oxygen concentration of the environment. In the case of *Planaria dorotocephala* certainly the mechanisms of respiration and circulation are, so far as known, simple and rudimentary; yet Hyman (1929) found that this form has a constant rate of oxygen utilization over a range of environmental oxygen tension between 3 cc. and 9 cc. per liter. If Krogh's suggestion has a basis in fact, one would expect that the water content of this animal is relatively high.

Planaria afford suitable material for a further study of the relation between water content and the degree of dependence of rate of oxygen consumption on the oxygen tension of the milieu. More specifically, the problem at hand is: What is the mechanism that renders the rate of oxygen utilization of this animal indifferent to changes in the oxygen tension of the surrounding medium? Krogh's suggestion indicates the point of attack, namely, the relation between water content and the effect of changes in oxygen tension on the rate of oxygen utilization with changes in free water content of the organism.

METHODS [1]

Investigation of this problem resolved itself into (*a*) finding the total free water content of *Planaria dorotocephala;* (*b*) increasing and decreasing the water content by means of hypo- and hypertonic solutions; (*c*) determining the rate of oxygen consumption of the animals in different concentrations of oxygen in natural water, and in hypo- and hypertonic solutions.

The free water content was determined by weighing the animals after removal from water, then drying over sulphuric acid in a desiccator, and weighing repeatedly until no further decrease in weight occurred. The first weighing was accomplished as follows: The animals were poured in water into a properly folded hard-surfaced filter paper, the water drained off, and the paper drawn across a plate of glass until it adhered firmly. The animals were then picked up with a thin spatula and placed in a weighing tube, the tube closed, and the weighing completed as rapidly as possible. The proceeding was timed so that the exposure of the animals to air was approximately the same in different experiments. Weighing the animals while wet introduces the error of the weight of water adherent to their surfaces. Considerable success was realized in standardizing this error, for during exposure to sulphuric acid the animals lost very nearly the same percentage of total weight in all experiments; which indicates that the original proportion of adherent water must have been much the same in each first weighing. The total loss of weight as shown by the data must be discounted by the weight of adherent water at the first weighing. The actual water content of the animals is therefore somewhat less than the data indicate.

The degree of dryness attained by desiccation over sulphuric acid depends on the relative affinities of the acid and of the animal tissues for water. The method was used by Davenport (1899) in his analysis of the changes in water content of the frog embryo during development. For the present work the method is more satisfactory than heating to dryness, for only free water is withdrawn from the organism. Heating to dryness involves loss of bound water as well as other substances. It is not assumed, however, that drying over sulphuric acid actually removes all the free water from the system, for the resistance of aquatic organisms to desiccation is common. However, the proportion of water remaining after the treatment must be very small; the residue is a brown mass that on pressure breaks up into a coarse granular powder.

[1] The experimental work on which this article is based was done in the Osborn Zoological Laboratories, Yale University. Acknowledgment is here made for the facilities and assistance provided.

The hypo- and hypertonic solutions employed throughout this work were distilled water and Ringer's solution made up with buffer and in concentration that is described as isotonic for amphibian tissue.

The effect of distilled water on the weight of *Planaria dorotocephala* and the analysis of the distilled water used are given in a former paper (Buchanan, 1930*a*). It was also shown that these animals in some way condition distilled water; accordingly in this work the proportions of animal tissues to distilled water were made approximately the same in all experiments. Since the changes in weight in hypo- and hypertonic solutions constitute an important phase of the problem at hand, the method employed in obtaining the data given in the former paper is restated.

Twenty-five animals of medium length were weighed and placed in 250 cc. of distilled water, then weighed again after certain intervals. The hydrogen ion concentration of the distilled water was reduced by the addition of a minute amount of NaOH. The experiments were conducted at room temperature, 20° to 23°. Control lots were weighed after similar handling in tap water and the difference in changes in weight between the distilled and tap water lots is regarded as due to the effect of distilled water on weight. The uniformity of the results was seriously interfered with by the fact that the distilled water causes the animals to exude slime. It is difficult to remove this slime during the weighing process. Furthermore, partial drying on filter paper and the incidental exposure to air causes a withdrawal of water from animals that have taken up water from a hypotonic medium. Thus the data show wide variations in weight changes in distilled water. It is clear, however, that after four hours in distilled water, pH 6.8 to pH 7.6, the weight increases. Consequently for respiration tests I employed animals that had been pre-treated with distilled water for from four to six hours.

The effect of Ringer's solution on the weight of *Planaria dorotocephala* is also given in a former paper (Buchanan, 1930*b*). Ringer's solution was employed because in the writer's experience the animals live longer in this hypertonic medium than in others that have been tried. At 20° medium sized *Planaria* will live for several days in Ringer's solution. Data on the effect of Ringer's on the weight of the animals were obtained by methods similar to those employed for distilled water. The results are fairly uniform, for the animals do not exude slime. It is shown that the weight decreases continuously during the first four hours of exposure, but after that time further loss of weight is slight. Hence, for tests of the effect of loss of water from the animals on their rate of oxygen consumption under different

conditions of oxygen concentration, I employed animals that had been exposed for four hours previously to Ringer's solution.

The oxygen content of the various liquids was determined by the Winkler method. This method has recently been criticised by Shearer (1930). His criticisms are based in large part on his statement that when present in numbers a European planarian discharges substances which should, according to Shearer, absorb iodine and thus interfere with the titrations. Shearer assumes that this possibility has not occurred to us. The fact is rather elementary that in iodometric work the possible absorption of iodine by substances in the solution must be taken into account. Years ago Hyman (1919) showed conclusively that the substances which *Planaria dorotocephala* excretes do not absorb iodine. Further evidence is to be found here. *Planaria* exude slime in distilled water but none at all in Ringer's solution, yet the titrations show that the oxygen consumption of the animals is materially less under comparable conditions in distilled water than in Ringer's.

Shearer also criticises work done with *Planaria* by this method on the grounds that the motor activity of the animals is not controlled. Shearer himself attempts to draw conclusions concerning the motor activity of an American flatworm which he probably has never seen, from quite unconvincing evidence obtained from a European form. If the head of *Planaria dorotocephala* is removed the animal continues to crawl about for some minutes. Then it comes to rest in a contracted state and if undisturbed does not change position for many hours. This is also true of *Planaria maculata* but not true of *Phagocata gracilis*. A comparison of the rate of oxygen consumption of beheaded *Planaria dorotocephala* with that of intact animals brought to rest by other methods, namely, lowering the temperature slightly and excluding light and other stimuli, shows no appreciable difference (Hyman, 1919*a*, 1919*b*; Buchanan, 1926). It has thus far been impossible in this, as in much of the work on standard metabolism, to exclude local muscular twitching and the action of cilia, without recourse to profound anesthesia. If the work on *Planaria* is invalidated by these internal and localized motor activities, then the same standards must be applied to a very considerable proportion of the work on respiratory metabolism, including Shearer's. In the present work the factor of motor activity of the animals is not seriously concerned, for the respiratory differences are of such nature and so conspicuously different that they cannot be explained on the basis of differences in motor activity.

The possibility of the salts in Ringer's interfering with the titrations

required safeguarding. Since only the relative quantities of oxygen in the medium before and after the animals had consumed some of it were of major importance, the action of salts may be discounted so long as it permits a sharp end point. To assure the accuracy of titration, a casserole containing the same quantity of Ringer's as the sample to be titrated was placed beside the casserole containing the sample. To this Ringer's was added sufficient iodine to match the color of the sample. It was then cleared with sodium thiosulphate. This served as a check end point, and the sample was then cleared to the same state of decoloration as the check. The end point in Ringer's was quite distinct and it was found unnecessary to provide this check as a constant procedure.

A much more difficult problem was the control of motor activity so that standard conditions obtained in the several environments. In part this was solved by the fact that *Planaria dorotocephala*, when changed from light to darkness, or when changed to a somewhat lower temperature, remain quiet for some hours. In this work both of these methods were employed. In both tap and distilled water the factor of motor activity does not enter, for in both the animals were quiet throughout the tests, except for the very brief time at the start, when the animals were distributing themselves about the interior of the flasks. In Ringer's solution the animals writhe for some time, then become paralyzed, accumulating at the bottom of the flask in abnormal positions. After two hours or less only occasional spastic movements occur unless light is admitted or the flask agitated. Unquestionably such twitching results in the consumption of oxygen. If one may judge from observations on the extent of such localized muscular activity, the amount of oxygen required for this movement must be very small as compared with the total oxygen consumption. Furthermore, there is no evidence in the observations that the extent of such movements increases with the increase in oxygen concentration of the Ringer's. After careful observation of the behavior of the animals as compared with the oxygen consumption under the several conditions, one must conclude that the differences in oxygen consumption cannot be ascribed to differences in motor activity.

In carrying out a titration the quantity of clearing agent used in reaching the end point is in practice slightly in excess or else slightly under the exact quantity. Also in the necessary manipulations no two lots of animals can be treated in exactly the same fashion. In order to compensate for such variations in procedure that result in a spread in the results, duplicate lots of animals were carried simultaneously in all experiments and the results recorded are the average

between them. The oxygen consumption of such duplicates varied in amount, sometimes rather widely. But with one or two exceptions both members were in the same direction from the control determinations and fell within ranges that are consistent with the general data.

If the oxygen concentration of the medium is reduced materially below saturation at room temperature, some oxygen will be absorbed during a two-hour experiment, from the air or from the wall of the flask, or by leakage through the connections. Likewise, if the oxygen concentration is higher than saturation at room temperature, oxygen will be lost during the experiment. Obviously, therefore, determinations of the oxygen content of flasks in which animals had been kept for two hours had to be corrected for changes in oxygen concentration not due to the metabolism of the animals. A considerable series of tests was made in an attempt to determine the magnitude of change at various oxygen concentrations. The apparent oxygen consumption of the animals was then corrected in each case in accordance with the average oxygen gain or loss at approximately the same oxygen tension in tap H_2O in the absence of animals. Admittedly this does not yield an absolutely accurate result, for the control tests just described showed that the amount of oxygen gain or loss is subject to some variation. However, this appears to be the most accurate and convenient method at hand for establishing the necessary correction. A tabulation of the average oxygen gain or loss at various oxygen tensions which was used as a table of corrections is given as Table III.

A typical experiment to test the effect of differences in osmotic pressure of the medium on the response of the organism to different oxygen concentrations was carried out as follows: Twenty-four hours before the test, if distilled water was to be used, a large quantity of distilled water was treated with carbon dioxide-free air, the water being treated for at least twelve hours. The H ion concentration was thus decreased to pH 7.0 prior to use in the experiment. A selected lot of animals was divided into two groups and their rates of oxygen consumption in tap water were taken over a two-hour period. In all cases the oxygen concentration of the tap water was at or near saturation at 20°; the H ion concentration being always lower than pH 7.0 but never as low as pH 7.8. The amount of oxygen consumed per two hours under these conditions is regarded as the normal and in comparison with the effects of other conditions is taken as 100 per cent. The animals were then placed in 500 cc. of distilled water, or of Ringer's, as the nature of the experiment required, the oxygen tension of which was approximately that of the tap water in the control test. After four or more hours a fresh supply with higher or lower oxygen

tension, but with the same H ion concentration, was siphoned into the flasks, after appropriate washings, which were then sealed and immersed in the constant temperature bath in darkness. Samples for titration were taken of the stock solutions and of the contents of the flasks at the end of two hours.

The oxygen concentration of the stock solutions was controlled either by bubbling pure oxygen through the stock, or by driving out most of the oxygen by heating and then cooling in a container so sealed as to prevent the ingress of air directly to the supply to be used. The time intervals were accurate to within one minute, the temperature within 0.5°, and the H ion concentration ranged in the various experiments from pH 7.0 to pH 7.8. In the case of Ringer's the pre-treatment with carbon dioxide-free air was not necessary, since the solution was buffered to pH 7.8. In all cases the test containers were Erlenmeyer flasks, capacity 500 cc., in which were placed from 35 to 60 animals, depending on size. Over a two-hour period the products of the animals increased the H ion concentration of distilled water approximately pH 0.2, somewhat less in tap water, and not at all in Ringer's. Therefore at no time was the H ion concentration greater than pH 6.8, nor less than pH 7.8. According to Hyman (1925), within this range the effect of H ion concentration on oxygen consumption is slight.

Data

Table I shows that the water content of *Planaria dorotocephala* is approximately 78 per cent of the total weight, as determined by drying over sulphuric acid. The accuracy of this value is of course conditioned by the weight of water adherent on the surfaces of the animals at the first weighing and by the distribution of water between the drying agent and the organic material. The affinity of the latter for water being undetermined, it is not justified to assume that all the free water is removed by this method. With these reservations, it is, however, certain that the water content of these animals is relatively low. Comparison of these data with those given by Davenport (1896, pp. 58–59) for other invertebrates shows that the water content of *Planaria* is comparable with that of other soft forms, although somewhat lower. It is much lower than that of hyaline forms referred to by Krogh (1916, pp. 58–59). Thus the observed facts do not support Krogh's hypothesis, that independence of rate of oxygen utilization of environmental oxygen supply is conditioned on a low content of dry substance.

The effect of distilled water and of Ringer's solution on the weight of *Planaria* is given in two former papers (Buchanan, 1930*a*, 1930*b*).

TABLE I

Loss of weight of Planaria dorotocephala over sulphuric acid. All animals starved two weeks before weighing. In milligrams and percentage change.

Length	No. of Animals	Live Weight	Final Weight	Loss
mm.		*mg.*	*mg.*	*per cent*
15	5	46.2	9.8	78
"	8	61.9	14.1	77
"	11	74.6	20.0	73
"	14	109.1	23.7	78
"	18	131.2	28.1	78
"	25	180.5	38.6	78
"	20	142.4	34.0	75
"	20	134.4	31.7	76
"	20	143.7	28.2	80
"	20 +	160.5	34.4	78
16	20	196.7	36.7	81
"	20	194.7	39.2	79
"	20 −	159.7	36.3	77
"	20 +	223.3	44.7	79

Table II is abstracted from the data there given and consists of weight changes in experiments in which the conditions were comparable to those which were employed in testing the effect of these environments on respiration. As stated above (p. 312), it was found impossible to weigh animals from distilled water with accuracy, due to the adherent slime and the accidental drying that occurs when the animals are handled on filter paper. The average weight gain shown by the data is approximately 15 per cent; the organism being 78 per cent water, this represents an increase in total free water of 11 per cent. No great confidence may be placed on these data, however. At best they merely indicate that water is taken up by the animal while in distilled water.

TABLE II

Changes in weight of Planaria dorotocephala after four- and five-hour treatment with Ringer's solution and distilled water. Abstracted from Table III, 1930*a*, and Table III, 1930*b*. All animals starved at least one week. 14–18 mm. animals. Twenty-five animals in 250 cc. of liquid in each case. Room temperature, Ringer's solution, pH 7.8; distilled water, pH 7.0 to pH 7.6. In percentage change as compared with controls in tap water.

Ringer's Solution		Distilled Water	
4 hours	5 hours	4 hours	5 hours
− 17.7	− 17.7	+ 1.9	+ 24.6
− 17.7	− 19.0	+ 3.7	+ 18.6
− 20.9	− 18.5	+ 4.5	+ 16.5
− 16.9	− 14.2	+ 1.9	+ 11.9
− 17.0		+ 0.6	− 2.8
− 10.0		+ 1.5	− 1.1
− 11.0			
− 12.0			
Average − 16%		+ 15%	

In the case of Ringer's, the data show clearly that the water loss amounts to approximately 16 per cent of the total weight, or a reduction of approximately 20 per cent of the total water content.

Figure 1 shows in graphic form the effects of changes in the oxygen tension of the medium on the rate of oxygen consumption in distilled

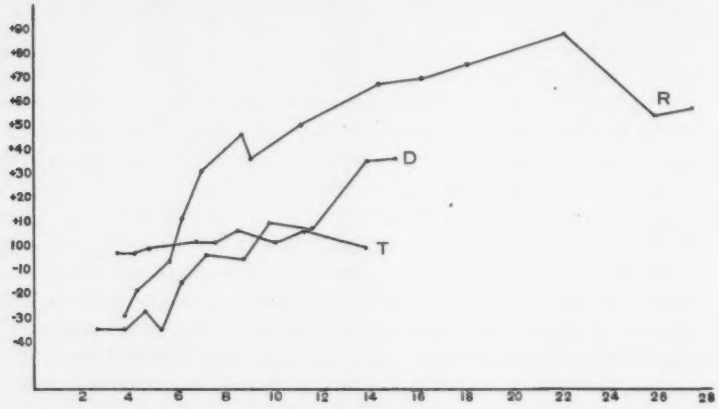

FIG. 1. The effect of increase in oxygen tension of the medium on the oxygen consumption of *Planaria dorotocephala*. Abscissæ represent cc. oxygen per liter. Ordinates represent changes in rate of oxygen consumption as compared with rate in tap water with oxygen concentration at or near saturation at 20°, which is taken as 100 per cent. *T*, animals in tap water; *D*, animals in distilled water; *R*, animals in Ringer's solution. Temperature, 20° ± 0.5°. H ion concentration, pH 6.8 to pH 7.8.

water, tap water, and Ringer's solution. The points on the respective graphs were determined by consolidating the data as follows: For each oxygen concentration duplicate lots of animals were used and the average between them is set down as the result at that particular oxygen concentration. Up to 8 cc. of oxygen per liter the determinations within a range of 1 cc. of oxygen per liter were averaged and plotted. Above 8 cc. per liter determinations of oxygen consumption within each concentration range of 2 cc. per liter were averaged and plotted. The graphs, then, represent 52 separate determinations of oxygen consumption in distilled water, 54 determinations in tap water, and 68 determinations in Ringer's solution.

The graph of the controls in tap water, *C* in Fig. 1, shows that within the range of oxygen tensions employed the rate of oxygen consumption does not change materially. For a number of years, in connection with other problems, data have accumulated on the rate

TABLE III

Average change in oxygen concentration in sealed 500 cc. flasks of tap water immersed in a water bath at 20° for two hours. Corrections for the data plotted in Fig. 1 were taken from this table. Change in percentage.

Range of Oxygen Concentration	No. of Experiments	Average Change
cc./liter		*per cent*
20–30	12	− 0.9
15–19	8	− 0.6
10–14	16	− 0.5
7–9	24	− 0.2
6	4	0.0
5	8	+ 0.1
4	4	+ 0.2
3	12	+ 0.6

of oxygen consumption of *Planaria dorotocephala* in oxygen tensions varying around the saturation point of water at room temperature. The data are in terms of the oxygen consumed per unit of wet weight per unit of time. At hand are 282 determinations that were made under comparable conditions; temperatures were between 18° and 24°; 208 experiments were conducted at 20°. The calculations necessary to determine the relationship between rate of oxygen utilization and the oxygen tension of the water have been carried out.[2] The results are as follows:

Number of experiments, 282.
Highest temperature of an experiment, 24° (3 cases).
Lowest temperature of an experiment, 18° (9 cases).
Average temperature, 20°.
Oxygen concentration of the water:
    Extremes, 3.90 and 7.19 cc. per liter.
    Mean, 5.87 cc. per liter.
    Standard deviation, 0.64 ± 0.018.
Oxygen consumption, per gram per two hours:
    Extremes, 0.30 cc. and 0.64 cc.
    Mean, 0.37 cc.
    Standard deviation, 0.05 ± 0.0014.
Coefficient of correlation, oxygen consumption and oxygen tension, 0.09 ± 0.04.

While a statistical treatment of data of this sort is not free from criticism, the fact that the coefficient of correlation is not significant, when considered together with the graphic results shown in Fig. 1,

[2] These calculations were made by Miss Rosalthea Sanders.

and the data of Hyman (1929), constitute convincing evidence that the rate of oxygen consumption of *Planaria* in natural water is independent of the oxygen tension over a wide range.

Figure 1 also shows that in distilled water after four to six hours the rate of oxygen consumption is sub-normal and remains fairly constant between oxygen concentrations of 2.65 and 5.35 cc. per liter.[3]

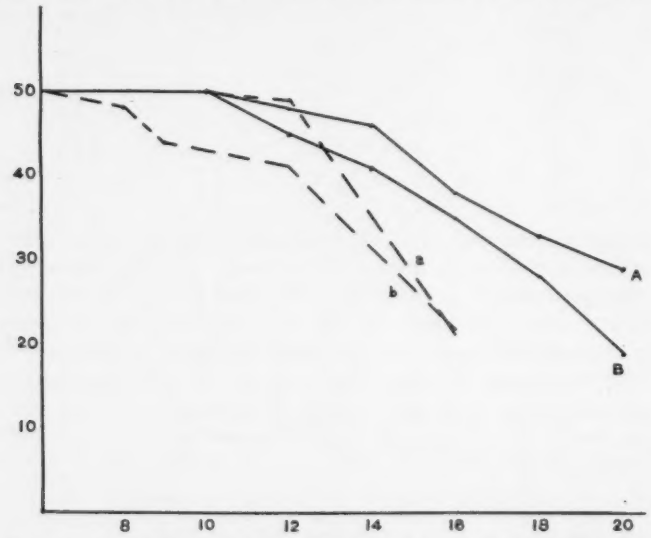

FIG. 2. Rate of disintegration of *Planaria dorotocephala* in distilled water with different oxygen content. Ten animals in 500 cc. distilled water in each case. Room temperature. Abscissæ represent hours; ordinates are derived from arbitrary values assigned to portion of animals alive at each observation, *e.g.*, 50 indicates that all 10 within a flask are alive; 40 indicates that some have partially disintegrated. *A* and *B* and *a* and *b* represent two different experiments. In *A* the oxygen concentration of the distilled water was 11.94 cc. per liter; in *B* the oxygen concentration was 6.16 cc. per liter. In *a* the oxygen concentration was 17.75 cc. per liter; in *b* the oxygen concentration was 6.51 cc. per liter.

It increases as the oxygen concentration is further increased, but is below that in tap water until the oxygen tension is increased beyond

[3] There is an apparent discrepancy between these results for distilled water and those of Hess (1929) and those given in an earlier paper by the writer (Buchanan, 1929*a*). The depressive action of distilled water shown here arises from the fact that the animals were pre-exposed, after washing with distilled water, from four to six hours, then washed by siphoning distilled water through the flasks for several minutes, then sealed in distilled water for two hours, thus bringing the total exposure to distilled water to totals of six and eight hours, in large volumes. The earlier results (1929*a*) and those of Hess (1929) agree that long exposure to distilled water lowers the rate of oxygen utilization. The data here given are not in conflict with those results.

9 cc. per liter. In Ringer's solution the fact appears that the rate of oxygen consumption is sharply dependent on the oxygen tension of the solution until the tension reaches a high value, approximately 14 cc. per liter. The effect of increasing the oxygen concentration above 14 cc. per liter becomes increasingly less; the data indicate that in the highest concentrations employed the rate of oxygen consumption is somewhat less than in lower concentrations.

Thus it appears that although the animals in natural waters are independent over a wide range of oxygen concentration, this is not a fixed condition, but is susceptible of being materially affected by either the salt content of the water, or by the osmotic pressure, or by both.

Figure 2 shows that animals in distilled water with a high oxygen content disintegrate slightly less rapidly than do animals in distilled water with oxygen content around the saturation point at room temperature. The method of plotting these data has frequently been described in detail and will not be re-described here. Reference to Fig. 1 shows that the rate of oxygen consumption of animals in distilled water containing corresponding concentrations of oxygen is higher in the higher concentrations, the reverse of the order of disintegration. In other words, in high oxygen concentrations the rate of oxygen utilization is greater while the rate of disintegration is lower than in normal oxygen tensions.

Discussion

With regard to the original problem, what is the mechanism that renders the rate of oxygen consumption of this animal indifferent to oxygen concentration changes in the environment, these results render certain hypotheses improbable.

In the first place, it is clearly shown that this characteristic, in this particular animal at least, may be modified by the action of salts and by the osmotic pressure of the environment. As a matter of methodology, this fact is of considerable importance. For if one were to measure the oxygen consumption of an animal in Ringer's solution, for instance, with the oxygen tension slightly greater than saturation at 20°, the data would appear to show that Ringer's accelerates oxygen utilization. On the other hand, should the oxygen concentration of the Ringer's be somewhat under saturation at 20°, the results would indicate that Ringer's is a depressant. The effects of various salts on respiratory metabolism have received considerable attention, but there is little uniformity in the findings of various investigators. A partial review of the literature is given by Hess (1929).

Secondly, the results are important in showing that the absolute size of the animals, *i.e.*, the surface-volume relation, as suggested by Shoup (1929), may be rejected as a general factor in determining the relation between rate of oxygen utilization and oxygen tension. Also the rôle played by the degree of perfection of the respiratory and circulatory mechanisms does not appear to be of general importance; it is inconceivable that Ringer's or distilled water in any way converts these mechanisms in *Planaria*.

With these hypotheses of limited application, attention must be focused on the cellular oxidative mechanisms, not sufficiently emphasized in Krogh's (1916) discussion. The possibility must be considered that the relation between rate of oxygen utilization and the oxygen concentration of the environment is conditioned on the concentration of oxidative enzymes in relation to the oxygen concentration within the tissues. In the total absence of any information whatever as to the oxygen tension in the tissues, the permeability of the tissues to oxygen, and the effects of Ringer's solution and distilled water on tissue permeability to oxygen, explanations of the causal factors in these results must be in large part conjecture. However, sufficient facts are shown by the data to justify a discussion of certain possibilities.

In the normal animal in tap water, assuming that oxygen is present and oxidizable substances are in excess in the tissues, the velocity of oxidations is a product of a constant and the concentration of the active enzymes. That is,

$$Velocity = kE.$$

So long as E remains constant, further increase in the oxygen tension should have no effect on the rate of oxygen utilization. This constitutes a possible explanation of the fact shown in Fig. 1, that increasing the oxygen concentration of the tap water has no effect on the rate of oxygen utilization within the concentration range employed.

But if the value of E is increased, thereby increasing the value of V, the oxygen concentration in the tissues becomes a limiting factor until it reaches a value equal to the oxygen-transferring power of the enzymes as conditioned by the rapidity of adsorption of the substrates. If the value of E is decreased, V is proportionately decreased and remains constant so long as the oxygen tension is adequate to maintain a constant adsorption rate.

The data on both Ringer's and distilled water offer some support for this interpretation, for it will be noted in Fig. 1 that when the oxygen concentration reaches a certain value the rate of oxygen

consumption tends to become constant. In distilled water this value is low, between 2.65 cc. and 5.35 cc. per liter, as would be expected if the cell enzymes were diluted. It is unfortunate that exact data on the water increase in distilled water have not been determined with accuracy; a dilution of 11 per cent could hardly account for the lowered rate of oxygen utilization as compared with the rate in tap water. Still, there is no general law stating the relation between enzyme concentration and the rapidity of an enzyme reaction except that small amounts of an enzyme are relatively more effective than are greater concentrations. The data here are in accord with this, but their nature does not permit of further analysis in this direction.

The evidence is considerably more impressive in the data on the effects of Ringer's solution. The rate of oxygen consumption tends to become constant at approximately 14 cc. oxygen per liter, a result to be expected if the cell contents are materially concentrated. Reduction of water content in Ringer's is approximately 20 per cent. These suggestions regarding the effects of both distilled water and Ringer's solution on the oxygen utilization necessarily include the assumptions that the oxygen tension in the tissues is less than that of the surrounding medium and increases with increase in environmental oxygen concentration.

Under normal conditions, with environmental oxygen in excess of requirements, undoubtedly one of the conditions which determine the rapidity of oxygen utilization in the organism is the concentration of the oxidative enzymes. It is not unreasonable to suppose, and the results here support the view, that at least one of the conditions that determine the degree of dependence of oxidative rate on oxygen tension of the milieu is the relative concentration of the oxidative enzymes.

Another interpretation of lines T and R in Fig. 1 is possible. For this, two assumptions are necessary: (*a*) that in tap water with oxygen concentration between the minimum and maximum employed, the intra-cellular oxygen tension is greatly in excess of requirements, and (*b*) that Ringer's materially reduces permeability to oxygen, thus reducing intra-cellular oxygen tension. Under such conditions the rate of enzyme-controlled oxidations within the cell in tap water may be expected to remain constant with increase in environmental oxygen supply, while in Ringer's increasing the environmental oxygen concentration would be expected to effect corresponding increase in intra-cellular oxygen tension, with consequent increase in rate of oxygen utilization. The fact that in Ringer's the effects of increasing oxygen concentration form a logarithmic curve rising well above the

rate in tap water must be referred to the effect of Ringer's in extracting water and by concentration increasing the rate of oxygen transfer by the enzymes.

However, the fact that the effects of increasing the oxygen tension in distilled water above 5.35 cc. per liter are in general parallel to the effects in Ringer's, in that the rate of oxygen consumption is no longer independent of the supply, seems to indicate that some condition common to both distilled water and Ringer's disturbs the normal oxidative mechanism. Apparently, the ability of the oxidative enzymes to transfer oxygen is not adversely affected, for in both the rate of oxygen utilization increases above the controls with increase in oxygen concentration. Since in time both distilled water and Ringer's are lethal, it is conceivable that this common effect is an indication of the onset of structural breakdown. The most probable early injurious effect that would bring about this common effect is interference with the normal cell permeability to oxygen. While permeability effects are usually referred to the cell boundary and there is substantial evidence that the cell boundary does regulate the admission of oxygen (Warburg, 1911), permeability of the general cytoplasm must also be considered.

An interesting fact appears in the data on the effect of increasing oxygen tension in Ringer's solution. When the oxygen concentration of the Ringer's is approximately at saturation for that concentration of salts at 20°, the rate of oxygen consumption is approximately that of normal animals in natural water. Oxygen being less soluble in Ringer's than in water at the same temperature, this saturation point therefore represents an environmental oxygen supply somewhat less than at saturation in tap water. The significance of this fact is not apparent; it may be merely incidental with this particular concentration of salts.

Figure 2 shows that oxygen in the concentrations employed is not toxic for *Planaria*. The animals disintegrate slightly less rapidly in abnormally high concentrations of oxygen in distilled water than in distilled water with oxygen tension around the saturation point at room temperature. Also, it may be pointed out, with reference to Fig. 1, that in distilled water at oxygen concentrations corresponding to those used in Fig. 2 the rate of oxygen consumption is higher in the higher concentrations, while Fig. 2 shows that the rate of disintegration is slightly lower in the higher oxygen concentrations. In other words, the higher the concentration of oxygen the less rapid the rate of disintegration and the higher the oxygen consumption. These facts substantiate the conclusion expressed in a former paper (Buchanan,

1930*a*), that disintegration in distilled water is not a direct result of the action of the hypotonic medium on the oxidative metabolism of the animal; that relative rate of disintegration in distilled water is not an index of relative rate of oxidative metabolism.

Summary

In tap water the rate of oxygen consumption of *Planaria dorotocephala* is independent of the oxygen concentration of the water between 3.53 cc. and 13.84 cc. per liter. In so far as the methods coincide, this confirms the results of Hyman (1929).

In Ringer's solution after four hours' exposure *Planaria* lose approximately 16 per cent of their total weight, or approximately 20 per cent of their total water content. In Ringer's the rate of oxygen consumption is sharply dependent on the oxygen concentration of the solution between 3.79 cc. and approximately 14 cc. per liter. Above this concentration the rate of oxygen consumption tends to become constant.

In distilled water the animals imbibe water; irregular data indicate that during the first four or five hours of exposure the weight increases on the average 15 per cent, which represents an increase in water content of approximately 11 per cent. The rate of oxygen consumption in distilled water is lower than normal and constant in oxygen concentrations between 2.65 cc. and 5.35 cc. per liter. Above this concentration the rate of oxygen consumption increases as the oxygen tension increases, rising above the normal at approximately 9 cc. per liter, and continues to increase as the oxygen concentration increases, up to 15 cc. per liter, the maximum employed.

Planaria dorotocephala contain approximately 78 per cent free water, as shown by weight loss on desiccation over sulphuric acid.

The facts indicate that two conditions may be involved in the regulation of the rate of oxygen utilization in relation to oxygen tension in natural water: Concentration of water within the organism, thus controlling the concentration of the oxidative enzymes; boundary regulation of the admission of oxygen. In this animal, size (surface-volume) and the degree of development of its circulatory and respiratory mechanisms do not appear to be important.

In abnormally high oxygen tensions in distilled water the rate of oxygen consumption is higher and the rate of disintegration is lower than in distilled water with oxygen tension at saturation at 20°.

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OBSERVATIONS ON *EUGLENA LEUCOPS*, SP. NOV., A PARASITE OF *STENOSTOMUM*, WITH SPECIAL REFERENCE TO NUCLEAR DIVISION[1]

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INTRODUCTION

A *Stenostomum* heavily infected with a colorless euglenoid parasite was found in a collection made near the University of Virginia. The morphology of this curious flagellate has led me to place it in the genus *Euglena* Ehrbg.

My purpose is to describe this new form, its nuclear division and its relation to its host; discuss its systematic position; and record some further observations upon its life history.

HISTORICAL

Euglena-like parasites of rhabdocoels have been reported on previous occasions. Haswell (1892) reported from an undetermined species in Australia an intracellular, colorless flagellate that had neither stigma nor flagellum. In 1907 the same author reported from the same continent another colorless form, from a mesostomid. It possessed a stigma and when liberated from the host, it no longer progressed by metabolic or euglenoid movement but became somewhat bottle-shaped and swam spirally by a flagellum. This parasite was found not only inside the cells but also in the space between the gut and body wall. He did not attempt to identify either of these forms.

In France, Beauchamp (1911) described *Astasia captiva*, a parasite within the "pseudocoele" of *Catenula lemna*. His description is fairly complete. This form retained its flagellum in the host and bore a colorless "rudimentary" stigma. It measured 30 to 40 microns in length and even when liberated from the host moved only by a rapid metabolic (euglenoid) movement. He speaks of, and his figures show, the oblique surface striations and spoon-like depression near the posterior end of the body. He mentions the "conduit buccal" and nucleus as being typical of the "Eugleniens."

[1] The writer is greatly indebted to Dr. B. D. Reynolds, under whose direction this investigation was carried out at the Miller School of Biology and the Mountain Lake Biological Station of the University of Virginia.

Materials and Methods

The infected *Stenostomum* (a new species soon to be described) was found in one of my aquaria in October 1929.[2] Although there were several other *Stenostoma* of the same, as well as of other species, in the aquarium, none were found to be infected. No free-living flagellates were found in the water that resembled the parasite. Other old aquaria in the laboratory as well as fresh collections were then examined. Of the hundreds of rhabdocoels examined, only two species were found infected and these were both species of *Stenostomum*. The other *Stenostomum* found infected is *S. predatorium* (Kepner and Carter, 1930). The infection is very rare in nature.

Fortunately, one of these *Stenostoma* was cultivated very easily in rather large glass dishes in which there were wheat cultures of small flagellates and ciliates. A half dozen of these flatworms added to a thriving protozoan culture will produce a hundred or more in two or three weeks. The worms were examined frequently to see if any of the free-living Protozoa had become established as parasites. Negative results are reported. Upon the addition of two or three infected *Stenostoma*, practically all the flatworms were infected in a week. In this way it was possible to have on hand as many specimens of *Euglena leucops* as were desired.

Various killing and fixing agents were employed. The best fixation of the chromatin was obtained with Carnoy's aceto-alcohol mixture. Absolute alcohol saturated with corrosive sublimate gave good results, as did Bouin's picro-formol solution, Allen's modification of the former, Schaudinn's and Zenker's fluids. Nuclear division was best studied in sectioned hosts, although smear preparations were also used. Heidenhain's iron alum haematoxylin was found to be the best stain for the nucleus, flagellum and blepharoplasts. The alcohol-xylol-paraffin method was employed.

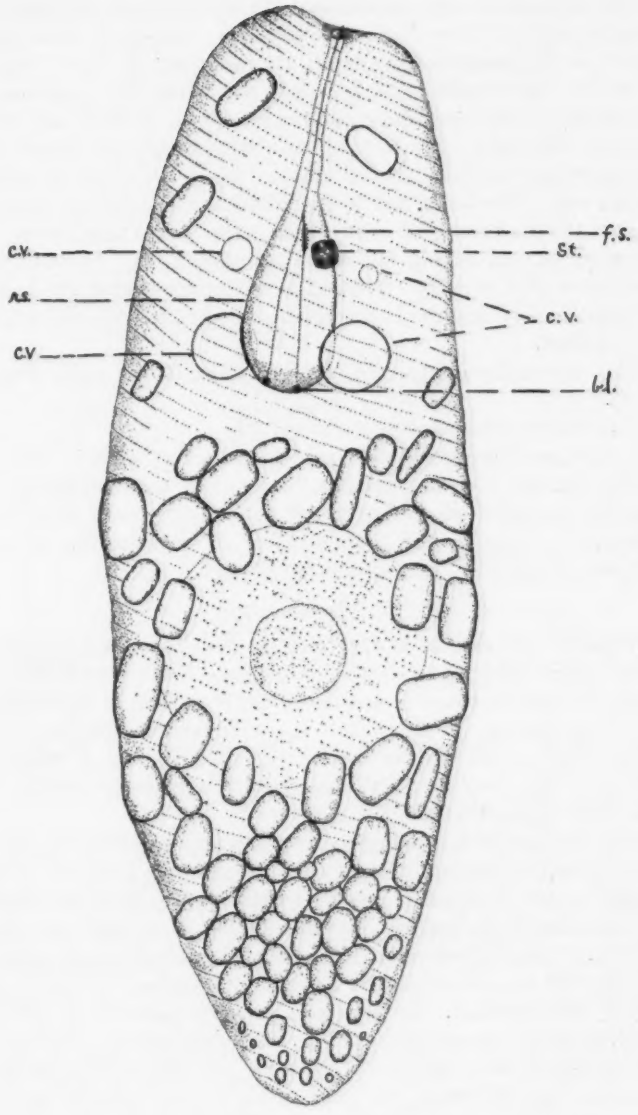
[2] I am greatly indebted to Dr. W. A. Kepner for help in the identification of the rhabdocoels employed in this investigation.

Plate I (× 8,000)

Fig. 1. Drawing of a non-flagellated living specimen of *Euglena leucops* sp. nov. as it appears immediately after liberation from the host.

The two rami of the flagellum can be seen to end at the blepharoplasts (*bl.*) at the base of the reservoir (*rs.*). The flagellar swelling (*f.s.*), a little anterior to the stigma (*st.*), is barely visible. The oblique surface striae are shown as well as both face and side views of the paramylum grains. Four contractile vacuoles (*c.v.*) are figured, two extended and two contracted. Details of the living nucleus other than the endosome can not be clearly made out. The stigma illustrated is composed of four distinct particles.

PLATE I



General Morphology

The organisms vary as to size. In a contracted condition the range in length is from 22 to 29.6 microns. Elongated, the range is from 33 to 44.5 microns. In width, the range is from 9 to 13 microns for resting individuals. In a resting condition the parasites are approximately two and one-half times as long as wide and rather blunt on both ends. The anterior end bears a slight depression in its contour where the gullet has its outlet. Most of a population are of average size. The forms just before cell division present the maximum size, but the daughter cells produced are often larger than others seen. The wave formed during metabolic movement may be as great in diameter as 18.5 microns. During metabolic movement the anterior end decreases only slightly in diameter while the posterior end becomes quite pointed.

Very fine surface striae run obliquely from left to right (Plate I, Fig. 1).

There are no chromatophores in the cell.

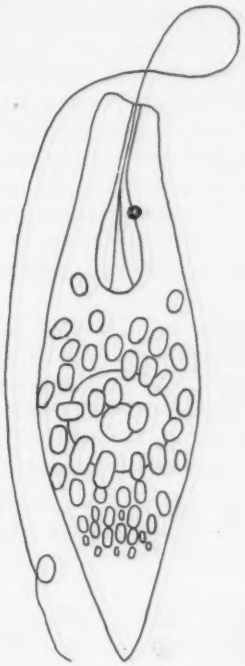
A gullet and reservoir can be seen in the living animal. The two together present a flask-shaped outline with a narrow passage, the gullet leading anteriorly. Associated with the reservoir are a series of contractile vacuoles that open into it. As many as four vacuoles have been counted in living specimens (Plate I, Fig. 1).

Flagellum

When the parasite is in the host the flagellum does not extend beyond the opening of the gullet (cytostome). Even in living individuals the flagellum can be seen to bifurcate as soon as it has passed the narrow passage of the gullet, the two rami proceeding to the blepharoplasts at the bottom of the reservoir. There is a "flagellar" swelling at the point of bifurcation, which is approximately at the level of the stigma (Plate I, Fig. 1).

When the parasite is liberated from the host and put into tap or spring water, a flagellum can be seen beginning to grow out of the "stump" in less than ten minutes. In twenty-five or thirty minutes in this medium it has reached its maximum length, a little longer than the body. Even before it has attained a length one-half as great as the body, the parasite ceases its metabolic movement and progresses feebly by the flagellum. In this condition the organism is dumb-bell-shaped, moving forward irregularly in very small spirals. The cell is still blunt on the posterior end at this stage. By the time the flagellum has reached its full length, the shape of the cell has changed greatly. It is now bottle-shaped, moving vigorously and swiftly by means of the newly acquired organelle of propulsion (Text figure 1).

The animal displays a marked faculty to replace a lost flagellum, since, as pointed out below, this structure when lost will be regenerated if the conditions of osmotic pressure are made favorable.



TEXT FIG. 1. Outline drawing of a flagellated specimen of *Euglena leucops* sp. nov.

Paramylum Bodies

These bodies are distributed irregularly over the cell, being most abundant in the posterior third. Only one to three small ones may be seen anterior to the stigma (Plate I, Fig. 1). These granules sometimes measure 4 microns at the greatest diameter. They are found to be disc-shaped and to decrease regularly when the parasite is subjected to inanition.

Stigma

The stigma, dark reddish-brown in color, stands out distinctly in the anterior region, just lateral to the gullet. It is not large, measuring less than two microns at the greatest diameter.

Much variation in size and form of the stigma has been observed (Text figure 2). The stigma is seen to be composed mainly of four to seven distinct bodies which appear to be connected by fine strands and the whole embedded in a colorless matrix. This morphologically degenerate stigma may represent a stage in the complete loss of this organelle.

A large number of observations were made upon the stigma of dividing individuals. While the nucleus is in the early prophase, the stigma enlarges slightly but usually the number of colored bodies of which it is mainly composed does not increase. These granules push apart somewhat and, keeping their identity, usually about one-half of them migrate, probably attached to each other, to the opposite side of the cell, never wandering far from the reservoir. They finally come to lie in a position lateral to the new gullet which has formed in the meantime.

This account of the origin of the daughter stigmata agrees somewhat with the observations of R. P. Hall and Jahn (1929*b*) on *Euglena gracilis*, except that they found that the stigma breaks up into a large number of smaller granules which become more widely separated.

The stigma does not always divide equally. A number of times the daughter cell has been seen to receive only two-fifths or even

TEXT FIG. 2. Variations in the stigma of *Euglena leucops* sp. nov. Drawings from several specimens. × about 16,000.

one-fourth of the dark granules. This unequal division aided by natural selection perhaps accounts for the decreased size and degeneration of the stigma.

Nucleus

The nucleus can be seen in the living organism as a relatively large spherical body (8 to 9 microns in diameter) lying usually near the center of the body. Upon fixing and staining, its chief characteristic is the presence of usually one comparatively large, deeply-staining, central body, the endosome (karyosome of some authors). Immediately surrounding the endosome is a hyaline area, apparently lacking in chromatin. The twenty-two to twenty-five chromatin bodies which

have been called "chromosomes" by Keuten (1895) and which give rise to the same number of chromosomes, remain distinct even in the nucleus of the interphase or the resting nucleus. Their distal ends lie close to and probably join the well-defined nuclear membrane. Fine strands are distinctly seen connecting the proximal ends of these bodies with the endosome (Plate II, Fig. 1).

These observations on the chromatin in the resting nucleus are not in agreement with those of most other workers on the euglenoids. Numerous small granules arranged at the nodes of a linin network have been described by Hartmann and Chagas (1910) for *Peranema*, by Tschenzoff (1916) for *Euglena viridis*, by Bělař (1916) for *Astasia*, by R. P. Hall (1923) for *Menoidum*, by R. P. Hall and Powell (1928) for *Peranema*, by Baker (1926) for *Euglena agilis* and by Ratcliffe (1927) for *Euglena spirogyra*. It is not difficult to see how the "numerous small granules" idea became prevalent, since this impression is frequently given by the nucleus after a casual observation, particularly following fixation in Schaudinn's fluid, the usual method employed. What I take to be the correct condition of the chromatin is better followed after the use of Carnoy's fixative. I have been able to observe the distinct "chromosomes" in the resting nucleus of *Euglena leucops* following fixation with all the agents mentioned in the section on materials and methods.

My observations are supported by Keuten (1895), who has figured chromosomes in the resting nucleus and in the late telophase for *Euglena viridis*. R. P. Hall (1925) has described the nucleus of the dinoflagellate *Oxyrrhis marina* thus, "Around the endosome the chromatin appears in the form of chromomeres, arranged in string-of-beads fashion in rows, or chromosomes; such an organization seems to be evident even in the resting nucleus, as characteristic of the dinoflagellates 'where there appears in the nuclei . . . to be a persistent organization of beaded chromosomes with subparallel or even spiral arrangements within the nucleus.'" [3]

In preparations fixed with Schaudinn's fluid and stained with iron haematoxylin, the endosome resists destaining very much longer than the chromosomes. After Carnoy's fixative and iron haematoxylin, the endosome decolorizes more readily than the chromosomes. The ability to destain the endosome almost completely has made possible a careful study of the resting nucleus and division stages.

A nuclear membrane is distinguishable in the resting condition and can be followed through mitosis.

Frequently, the endosome is seen to be in two, three or more

[3] Hall is quoting from Kofoid (1923).

fragments. R. P. Hall (Hall and Powell, 1928) noticed a multiplication of the endosome in *Peranema trichophorum*. Wenrich (1924), in his description of *Euglenamorpha Hegneri*, says: "In the pellucid variety there is a marked tendency for the nucleus to hypertrophy. . . . This hypertrophy is accompanied by a multiplication of the caryosome, as many as four have been found in one nucleus. Hypertrophy apparently leads to amitotic division of the nucleus which is probably followed by division of the body. Such amitotic stages have not been found in the green variety." No explanation is offered here for fragmentation of the endosome occurring in *Euglena leucops*. However, no evidence whatever for amitosis was found.

Behavior

Euglena leucops is stimulated to extremely vigorous movement by light, heat, and a change either way in osmotic pressure.

Thigmotropism is exhibited in non-flagellated individuals. Flagellated organisms were found to be negatively phototropic.

Plate II (× 10,000)

All the figures were drawn from specimens fixed in the host with Carnoy's aceto-alcohol mixture and stained with Heidenhain's iron alum haematoxylin.

Fig. 1. A typical nucleus of the interphase. Twenty-two chromatin bodies or "chromosomes" are figured. Note the attachment of these to the endosome.

Fig. 2. A very early prophase. The "chromosomes" and endosome are beginning to enlarge.

Fig. 3. The nucleus is becoming noticeably larger and the "chromosomes" and endosome are beginning to elongate.

Fig. 4. Note the blunt bifurcation of one end of the endosome.

Fig. 5. The nucleus, chromosomes and endosome are all distinctly elongated. Most of the chromosomes have apparently lost their attachment to the endosome.

Fig. 6. This is the first stage in the metaphase. The chromosomes are quite narrow and extend the length of the elongate nucleus. The blunt bifurcation of one end of the endosome is distinctly pronounced by now and both ends have become enlarged.

Fig. 7. Metaphase and the beginning of the anaphase. The chromosomes, having spun out practically the length of the elongated nucleus during the early metaphase, now divide transversely as the extremities of the nuclear membrane and endosome further pull apart. Note the constriction in the membrane and endosome. Fine strands are seen in the mitotic figure at this stage, most of which appear to be a connection between the two broken ends of the chromosomes.

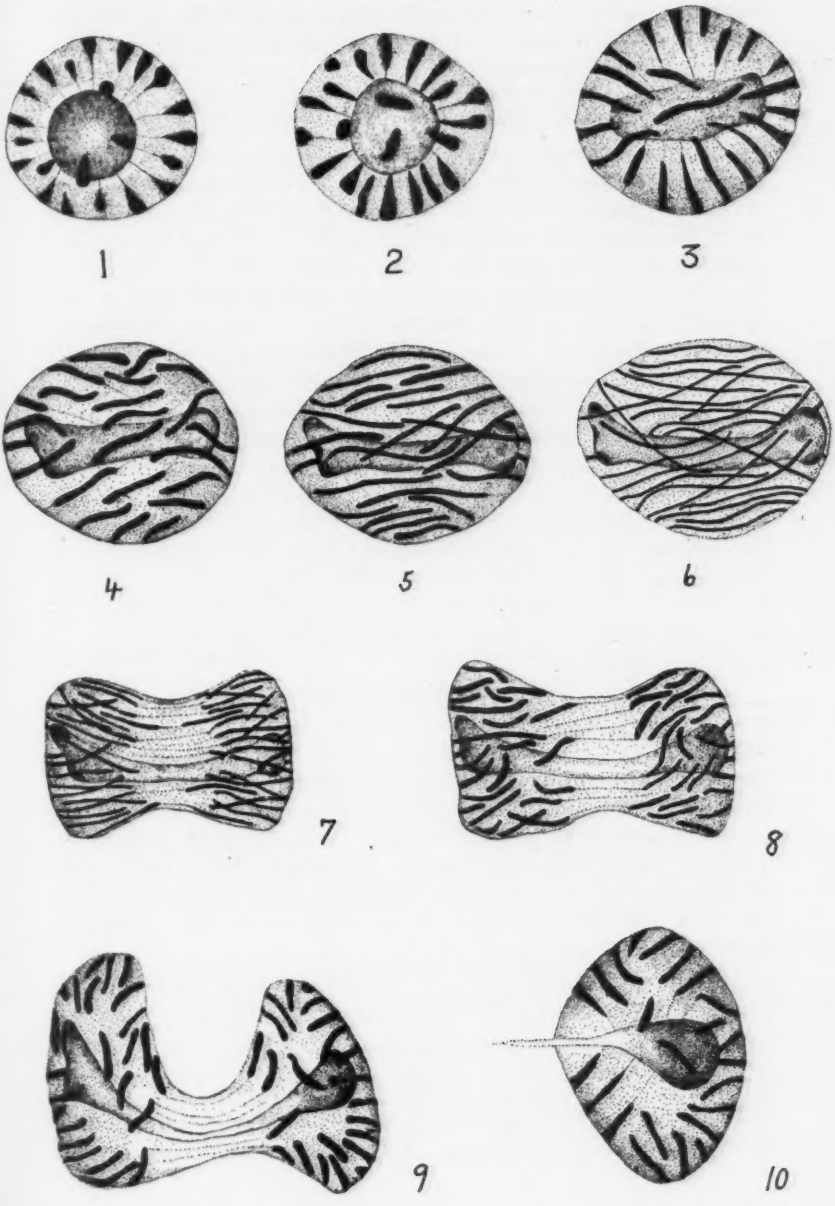
Fig. 8. The chromosomes are beginning to round off somewhat and are apparently attracted toward the two ends of the membrane. The nucleus and endosome are now decidedly elongated and constricted in the center.

Fig. 9. The chromosomes are beginning to assume their interphase shape and their attachment to the ends of the dividing endosome again becomes evident. Notice the upward turn of the nuclear membrane and endosome.

Fig. 10. The end of the telophase. Most of the chromosomes have regained their attachment to the endosome. The daughter nucleus and endosome now round off as the interphase approaches.

I am indebted to Miss M. E. Hill for help in the preparation of the drawings.

PLATE II



The fact that *Euglena leucops* elaborates a flagellum soon after being liberated from the host and is negatively phototropic,—the host reacts similarly—makes it particularly well adapted for swimming away and finding a new host.

Division of the Body

When an infected host is macerated in spring water, at any hour of the day, numerous dividing parasites are encountered after a few minutes. Frequently as many as 10 per cent of the flagellates, after ten to fifteen minutes exposure to this solution, will start dividing and complete the process within seventeen to twenty minutes. Now if an infected host is macerated in a solution approximately isotonic with the host's plasma, no more dividing forms are seen in this medium than normally appear in the host and twenty-five to thirty minutes are required for the parasite to complete the division. Since the spring water is of a lower molecular and ionic concentration than the host's plasma, it would appear that the change in osmotic pressure both initiates and accelerates cell division.

Division in *Euglena leucops* seems to be periodic, as is the case in *Hydramoeba hydroxena*. Reynolds and Threlkeld (1929) observed that division in this amoeba occurred most often about 3 A.M. Observations upon more than three hundred dividing forms of *Euglena leucops* have shown that division, while sometimes occurring at other times, most often takes place between 10 P.M. and 2 A.M., with the peak about midnight.

A short time before the longitudinal split occurs, there is a duplication in the number of gullets, reservoirs, flagellar "stumps," stigmata, and nuclei. The split begins at the anterior extremity and continues posteriorly along the median plane of the body until the two daughters are completely separated. There is a continual twisting and writhing of the binucleate body during the process of division. Finally the posterior ends of the daughter cells are connected only by a small strand, but this is apparently the toughest part of the cell, for a veritable tug of war ensues, before the daughter cells are separated.

The parasite has been under observation in the host for more than a year and no evidence of encystation has been observed.

Nuclear Division

Prophase

The beginning of nuclear division may be recognized in four ways: (1) increase in size of the nucleus, (2) migration of the nucleus anteri-

orly, (3) changes in the endosome and (4) changes in the chromatin bodies or "chromosomes."

A considerable increase in the size of the nucleus is observed at the onset of division.

By the end of the prophase the dividing nucleus has reached a position in the anterior part of the organism, just posterior to the reservoir.

The endosome increases in size and elongates. One end can be distinguished from the other by a blunt bifurcation (Plate II, Figs. 4, 5, 6, 7, 8 and 9). Baker (1926), working with *Euglena agilis*, describes the bud arising from one of the lateral points, as forming the blepharoplast of one ramus of the new flagellum and the connection of the bud with the endosome as the rhizoplast. I find insufficient evidence from *Euglena leucops* to support this.

By the end of the prophase the endosome is distinctly dumb-bell-shaped (Plate II, Fig. 5). It would appear that at the end of the prophase there is a brief pause in mitosis since this stage was encountered most often.

The distinct, rather large chromatin bodies or "chromosomes" of the resting nucleus, keeping their identity, increase slightly in size and stain more deeply early in the prophase (Plate II, Fig. 2). As the nucleus and endosome elongate, these bodies, with one end apparently remaining attached to the nuclear membrane, are spun out into extremely long chromosomes which become arranged around the endosome (Plate II, Fig. 5). The connection between the chromatin bodies or "chromosomes" and nuclear membrane persists certainly to middle prophase and perhaps until division is completed.

Metaphase

The endosome continues to elongate, its ends increasing in size at the expense of the middle portion (Plate II, Fig. 6). One end still can be distinguished from the other. The nuclear membrane follows largely the contour of the endosome. The chromosomes, having drawn out almost the length of the elongated nucleus, now break in the middle.

There is apparently little doubt that the chromatin bodies of the resting nucleus give rise directly to the same number of elongated chromosomes that divide transversely. Twenty-two to twenty-five bodies in the resting nucleus can be followed through the metaphase when the long chromosomes produce a double number of daughter chromosomes of approximately half the length of those in the late prophase (Plate II, Fig. 7).

I have been unable to find the V-shaped chromosomes of the late prophase that have been described for euglenoids by R. P. Hall (1923, 1928), Baker (1926), and Ratcliffe (1927) which they take as evidence for a longitudinal split.

After a review of a large part of the literature on mitosis in the Protozoa, I have been able to find only one other case where a transverse division in the chromosomes has been definitely demonstrated, although others have mentioned it as a possibility [Bělař (1916) in *Astasia* and Borgert (1909, 1910) in *Aulacantha* and *Ceratium*]. Calkins (1929) describes a transverse division in the chromosomes of the ciliate *Uroleptus Halseyi* on the third meiotic spindle.

Anaphase

During this stage the ends of the endosome become quite far apart but remain connected by their narrow, middle portion. The bend is quite pronounced, the two ends of the endosome making a sharp upward turn.

The forty-six to fifty daughter chromosomes now shorten but remain radially arranged around the extreme ends of the daughter endosomes. The membrane has become distinctly constricted in the middle largely following the contour of the endosome (Plate II, Fig. 8).

Telophase

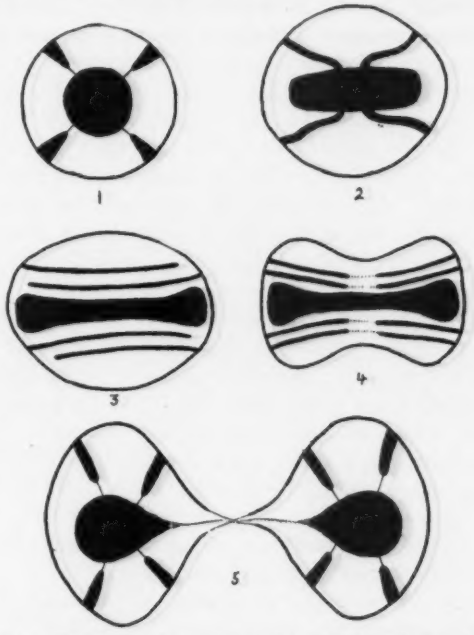
This stage begins when the ends of the daughter endosomes and daughter nuclei have pulled apart. The original endosome breaks in the center of its now narrow, lightly-stained, central portion.

The daughter endosomes and nuclei now round off and the chromosomes arrange themselves at the periphery of the nucleus. The connection between chromosomes and endosome is established early in the telophase (Plate II, Figs. 9 and 10).

The accompanying diagram (Text figure 3) illustrates the history of four chromosomes, in the resting nucleus and through mitosis. Kater (1926, 1927, 1928) brings forth evidence that even in the higher plants and animals, reconstruction of the daughter nuclei is by vesicle formation of contiguous chromosomes, that may be traced well into the succeeding prophase. He considers this as "good evidence for genetic chromosomal continuity." The evidence for this continuity is still stronger in *Euglena leucops*.

Calkins (1929) has the following to say concerning the chromosomes of the ciliate *Uroleptus Halseyi:* "In *Uroleptus Halseyi* the chromosomes are divided transversely at each vegetative division. On the theory of the gene these facts are possible only on the assumption of

a single type of gene for each chromosome and on this assumption there would be not more than twelve types of genes in *Uroleptus Halseyi*. Here there are twelve chromosomes in the third meiotic spindle and twenty-four in the amphinucleus. There are forty-eight in the first meiotic spindle and twenty-four in the second. We conclude that each of the twenty-four chromosomes found in the amphinucleus divides once to form the forty-eight of the first meiotic spindle; that two of these are separated from two by this division, and that one is separated from one at the second meiotic division, thus leaving twelve in the third meiotic spindle in which each chromosome represents a single type of gene. It is immaterial, therefore, whether division of each chromosome is transverse or longitudinal,



TEXT FIG. 3. Diagrammatic representation of the resting nucleus of *Euglena leucops* sp. nov. and the history of four of its chromosomes during division.

Fig. 1—the resting nucleus.

Fig. 2—the middle prophase.

Fig. 3—the beginning of the metaphase.

Fig. 4—the end of the metaphase and the beginning of the anaphase.

Fig. 5—the late telophase wherein begins the reconstruction of the nucleus of the interphase.

See the text and Plate II for a more detailed description.

for it would be equational in either case." Carrying this theory a step further, since the chromosomes are distinct even in the resting nucleus in the case of *Euglena leucops*, a single type of gene can be considered as recognizable at any stage in the life of the organism.

Experiments on Retardation in Growth of the Flagellum

As already stated, the flagellum of organisms liberated into tap or spring water will make its appearance outside the gullet in five to ten minutes. In 12 per cent Locke's solution, the organisms have been carried fourteen days without ever elaborating a flagellum. In a pure glucose solution with a molecular concentration approximately equal to the Locke's solution they have been carried more than a day or until death without showing a flagellum. The same thing is true for creatine, a mixture of different amino acids and other soluble protein derivatives. By varying the concentration of the molecular or ionic substances it is possible to vary the time required for the flagellum to be elaborated and for the appearance of the changes in body form that accompany it. The hydrogen ion concentration apparently has nothing to do with the retardation in the development of the flagellum. Viscous substances such as thin starch paste, as well as aqueous agar mixtures, which of course do not alter the molecular concentration of the media appreciably, have little or no effect on retarding the growth of the flagellum.

By increasing the osmotic pressure of the medium in which *Euglena leucops* elaborates and retains the flagellum, the organisms have been made to settle down and again move only by metabolic movement, the flagellum apparently being broken off. But if the osmotic pressure be once more decreased, the flagellum will reappear. This process has been repeated four times with the same individuals and the organelle grew out as readily the fourth time as it did the first.

It would appear therefore, that molecular or ionic concentration is the main factor in retardation of flagellar growth and that this organelle is capable of continued or repeated growth.

The osmotic pressure of the plasma of the host is apparently great enough to prevent the elaboration of a flagellum.

Results of Attempts to Cultivate the Parasite

All attempts to cultivate the parasite *in vitro* failed. They were carried fourteen days in Locke's solution diluted to 12 per cent at pH 7.6. At the end of this period practically all the paramylum was consumed.

They may be kept alive three or four days in spring water.

Methods of Infection

The parasite may enter the host in three ways:

(1) By the ingestion of liberated parasites. The *Stenostoma* that the writer has been able to infect, feed extensively upon Protozoa. The parasites, either swimming actively or moving on the bottom of the container by euglenoid movement, are ingested by the host and enter the space between the gut and body wall, presumably, directly through the wall of the enteron.

(2) The *Stenostoma* susceptible to infection are predatory and cannabalistic. On a number of occasions I have observed the transmission of the infection by an infected flatworm serving as food.

(3) Vegetative zoöids are infected from the parent.

The Effect of the Infection upon the Host

Upon one occasion the writer observed an uninfected *Stenostomum* ingest a portion of an infected one of the same species. About eight parasites entered the enteron of the uninfected rhabdocoel. In less than an hour approximately all of the parasites were seen in the mesenchyme of the new host. The next day they had about doubled in number. The host divided on the second day and the (approximately thirty) parasites were distributed about equally between the two. At the end of a week the infection had become well established.

Stenostoma lightly infected show no ill effects whatever and appear normal in all their reactions. No signs of "nervousness" are exhibited even though the parasites can be seen passing over, under and in contact with the cephalic ganglia. Kepner and Carroll (1923) found this to be true in the case of *Stenostomum leucops* infected with the ciliate, *Holophyra virginia*.

Since the parasites evidently absorb their food from the plasma in the mesenchyme, one would expect that rather heavily infected animals would grow and reproduce slowly; but specimens infected with as many as fifty or sixty parasites have been seen to divide every second day under favorable conditions. This is as fast as normal individuals reproduce.

Frequently the flatworms become infected with two or three hundred parasites which causes them to appear bloated, due to the ectoderm being lifted to make room for the increased contents of the mesenchyme. Animals in this condition become sluggish, the ectoderm breaks in one or more places, the parasites are liberated and death generally follows in a day or so.

Means for freeing the flatworms of the parasites have not been found.

Infection Experiments

Even though species of *Stenostoma* other than the two mentioned above ingest the parasite, only one, *S. grande*, has been infected experimentally. All attempts to infect *Catenula lemna*, the host of *Astasia captiva*, failed, even though it ingested *Euglena leucops* in large numbers.

Systematic Position of Parasite

The apparent specificity of this parasite excludes the probability of its being found in hosts other than rhabdocoels. The only euglenoid described as a parasite of this group is *Astasia captiva*, from *Catenula lemna*, and this rhabdocoel apparently is not capable of becoming infected with *Euglena leucops*. *A. captiva* differs further from *E. leucops* in the following respects:

(1) *A. captiva*, according to Beauchamp (1911) is without a stigma, save possibly a *colorless* rudiment.
(2) It has a flagellum while in the host.
(3) It does not change in shape or otherwise when freed into spring water.
(4) *A. captiva* dies in a few hours after liberation.
(5) The paramylum grains of Beauchamp's parasite are elliptical in outline.

The *Euglena*-like form that Haswell (1907) mentioned but did not describe, resembles very closely *Euglena leucops*. Nevertheless it was intracellular and a parasite of a mesostomid, the most highly specialized of the Rhabdocoelida, and hence the farthermost removed from *Stenostomum*.

Wenrich (1924) has shown that the colorless variety, *pellucida* of *Euglenamorpha hegneri* may arise from the green variety, simply by loss of color. More conclusively Zumstein (1900) and Ternitz (1912) have shown that *Euglena gracilis* will lose its chlorophyll and become colorless when supplied with rich nourishment. They point out that their results do away with the boundary line between the genera *Euglena* and *Astasia* which have been separated on the basis of color (chlorophyll and stigma). Color, therefore, is not a sound character on which to base a classification of *Euglena*-like forms.

R. P. Hall and Jahn (1929*a*) say "A bifurcation of the flagellum is characteristic of the different species of uniflagellate Euglenidae examined and the flagellum in such species always shows a 'flagellar swelling' at the level of the stigma (which is always present) in vegetative stages. . . . Such structural features, however, have not

been observed in the vegetative stages of any of the non-chlorophyll-bearing species examined by us or by other workers."

Further, they believe that certain stigma-bearing but chlorophyll-free flagellates, described in the literature as *Astasia*, should be put in the genus *Euglena*. They made no observations upon the conditions of the flagellum in these forms.

Bělař (1916) was of the opinion that *Astasia captiva* was an *Euglena*. He possibly was influenced by the presence of a stigma in this form, even though Beauchamp states that it was a colorless rudiment.

Because of the presence of a definite stigma and the bifurcation of the flagellum, the writer is of the opinion that this new form should be placed in the family Euglenidae Stein. If it is placed in this family, obviously it belongs in the genus *Euglena* Ehrbg. because of its morphological features, which would preclude its belonging to any other genus of this family.

Summary

A new species of chlorophyll-free *Euglena*, *E. leucops*, is described, and though it lacks color, the presence of a stigma and the bifurcation of the flagellum place it in the family Euglenidae.

A flagellum is not present while the parasite is in the host but is elaborated quickly outside the host if favorable conditions of osmotic pressure prevail. This organelle is capable of repeated growth.

The stigma has been followed through binary fission and found to divide, but not always equally.

Nuclear division is described. The "chromosomes," which remain distinct, even in the resting nucleus, divide transversely during the metaphase.

Osmotic pressure apparently may initiate and accelerate cell division.

Attempts to cultivate the parasite *in vitro* failed.

The host may be easily cultivated and the infection readily continued.

No evidence of encystation has been observed.

Observations upon the flagellates for more than a year have given no evidence of a method of division other than binary fission involving mitosis.

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ALTERNATION OF GENERATIONS IN THE ROTIFER *LECANE INERMIS* BRYCE

I. Life Histories of the Sexual and Non-sexual Generations

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(From the Zoological Laboratory of the Johns Hopkins University)

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The Organism

The small loricate rotifer, *Lecane* (*Distyla*) *inermis* Bryce, presents many advantages for the study of the alternation of parthenogenetic and bisexual generations, common among the rotifers, and of the life histories of the diverse types of individuals that occur. It is hardy, easily cultivated, multiplies rapidly and changes readily from the non-sexual to the sexual condition and vice versa. The entire life of the female lasts usually but nine or ten days, and the first offspring appear on the second or third day. This organism is of interest as a representative of a genus in which the occurrence of males has not

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hitherto been described (Wesenberg-Lund, 1930, p. 94). The male of this species was first observed by Finesinger subsequent to his study (1926) of the inheritance of certain environmental effects in a succession of generations of parthenogenetic females.

Lecane inermis is one of the slow-moving rotifers, common in wet sphagnum. It belongs to the family Euchlanidæ, which is characterized by the possession of a lorica composed of two parts, a dorsal and a ventral plate, connected by lateral sulci. In *Lecane inermis* the lorica is extremely flexible, so that dorsal and ventral plates are barely distinguishable; the animal therefore resembles somewhat the more primitive, soft-bodied notommatids, with which the euchlanids are classified by Wesenberg-Lund (1929). A full description of this and other species of *Lecane* is given by Harring and Myers (1926). The individuals (females) of the stock employed in the present investigation are somewhat larger than the dimensions given for this species by Harring and Myers. Adults are 175–190 μ in length by 65–75 μ in breadth; newly-hatched individuals are about 130 μ in length by 33 μ in breadth.

As is well known, the rotifers of most common occurrence are the parthenogenetic or amictic females, and specific descriptions are based, in large measure, on these. In a single species there may occur, however, three sorts of individuals: the common amictic or non-sexual females, the mictic or sexual females, and the males. An outline of the change of generations will facilitate the understanding of the present study.

Outline of the Change of Generations

(Compare Fig. 1)

The common non-sexual or amictic females (*A*) multiply exclusively by parthenogenesis. They deposit eggs (*f*) that are ellipsoid in form, with a thin, smooth, outer membrane. These eggs carry the diploid number of chromosomes, are incapable of fertilization and produce females, usually like the mother, so that multiplication by (diploid) parthenogenesis may continue for many generations. But among the females produced by such eggs are at times individuals (*M*) that bring forth small eggs (*m*) which develop into the small, male individuals. These females are called mictic because their eggs are capable of being fertilized. They resemble outwardly the amictic females. The small, male-producing eggs carry the haploid number of chromosomes. There is some evidence that the male embryo may either double its chromosomes during development (*Asplanchna intermedia*, Tauson, 1924) or develop entirely with the haploid number (*Asplanchna amphora*, Whitney, 1929). When the mictic females are

fertilized they produce, instead of the small, haploid, male-producing eggs, larger, dark eggs with a thick, horny shell. Such an egg has, of course, the diploid number of chromosomes. It remains unhatched for some time, and then produces a parthenogenetic or amictic female,

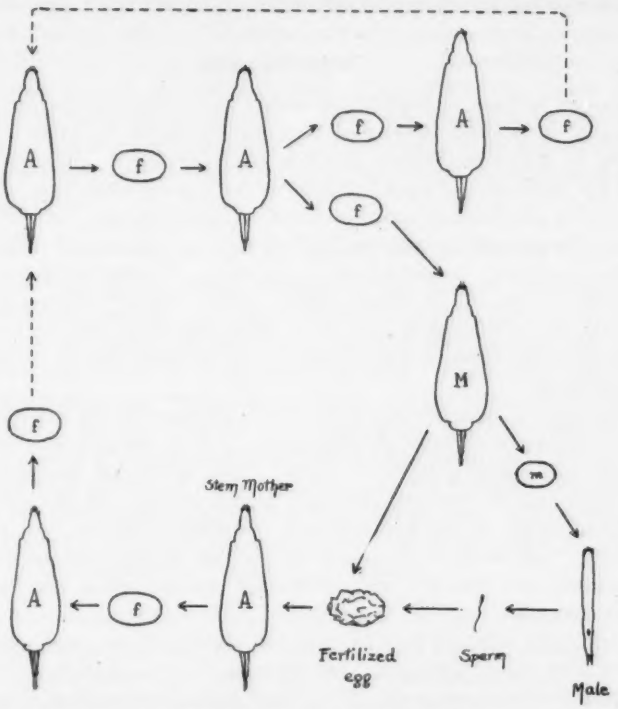


FIG. 1. Diagram showing the alternation of sexual and non-sexual generations in a bisexual rotifer, such as *Lecane inermis*. The most common type of individual, the non-sexual "amictic" female (*A*), reproduces exclusively by diploid parthenogenesis. Its eggs (*f*) develop invariably into females, which are usually amictic, so that multiplication by diploid parthenogenesis may continue for many generations (as indicated by the long, broken-lined arrows). But the daughters of the amictic female may be "mictic" (*M*). Their eggs are haploid, and develop parthenogenetically (*m*) into males, or, if fertilized, produce non-sexual amictic females, the "stem mothers," with which the cycle begins anew.

with the diploid number of chromosomes. From such a female, commonly spoken of as the stem mother, begins again the cycle of diverse generations just described.

The purpose of the present investigation is to analyse this cycle in the species *Lecane inermis;* to give an account of the different

types of individuals composing it, and to discover the factors that cause their production. This first paper presents a description of the different types of individuals, with a comparative study of their life histories. A later paper, now in preparation, will deal with the factors causing the change of generations.

Culture Methods

Lecane inermis is readily cultivated in grain infusions of proper strength, in malted milk, and in some standard inorganic culture media to which are added suitable food organisms. Oat infusion, used by Jennings and Lynch (1928—I) for *Proales sordida*, is (as they observe) a satisfactory medium also for *Lecane inermis*. An infusion of suitable strength is prepared by boiling twenty flakes of rolled oats for three minutes in 100 cc. of spring water, filtering while hot, and allowing to stand 24 hours before using. This "standard" oat infusion was employed in the comparative studies of the life histories of the different types of individuals and in the early part of the experimental work.

For precise experimental study of the influence of the environment on the kinds of individuals that occur, a more exactly controllable culture medium is necessary. Luntz (1926, 1929) has demonstrated the usefulness of synthetic inorganic media, such as the Benecke and Knop solutions, in the experimental investigation of the life cycle of rotifers. The food is controlled by the use of pure strains of food organisms, and the pH and initial salt concentration are likewise under control.

Benecke's solution in 0.07 per cent concentration, with addition of suitable food organisms, provides a most satisfactory culture medium for *Lecane inermis*. To it were added pure strains of certain bacteria, *B. proteus*, *B. subtilis* and *B. coli* (obtained from the School of Hygiene of the Johns Hopkins University). Such pure strains are inadequate as food, the rotifers surviving in them for but three generations. A mixture of two or three of these strains of bacteria is more satisfactory. Addition of green algæ to the bacteria gives still better results. A number of different algæ, obtained from the laboratory of Pringsheim at Prague, were tested. Species of *Chlamydomonas* and *Euglena* were apparently too large to serve as food for *Lecane inermis*. *Chlorella vulgaris* Beyerinck proved to be suitable, particularly when combined with *B. proteus*. The mixture of *Chlorella* and *B. proteus* has been employed in much of the experimental work. *Chlorella* is grown on beef agar, *B. proteus* on bacteriological agar.

A fresh suspension of these two food organisms in the culture solution is prepared daily immediately before the transfer of the rotifers.

In order to avoid contamination with other organisms, bacteriological precautions are observed in the preparation of the culture medium and food suspension, and in the handling of the rotifers. The method is, with a few necessary alterations, that devised by Raffel (1930) for the culture of *Paramecium*. The rotifers are cultivated individually on hollow-ground slides, placed in sterile Petri dishes containing distilled water. They are transferred daily with sterile, cotton-stoppered pipettes. A fuller account of the method of culture will be given in a later paper on the factors causing the change of generations.

Life Histories of the Diverse Types of Individuals

Knowledge of the general biology of the bisexual generations of rotifers is not extensive. This is doubtless due in part to the rare occurrence of sexual individuals in many of the species that have been employed for laboratory investigations. There have been few detailed comparative studies of the lives and activities of the mictic and amictic females, though such studies are much needed for an understanding of the fundamental difference between them, and of the different rôles they play in the life history of the species. Knowledge of the life history of the males is in little better case. *Lecane inermis* is a favorable organism for supplying these needs, since it readily yields an abundance of all types of individuals.

The Amictic and the Unfertilized Mictic Females: Comparison

The amictic and mictic females of *Lecane inermis* resemble each other closely, but differ markedly in certain features of their life histories, particularly in fecundity and length of life.

The mature females, whether amictic or mictic, present the appearance shown in Fig. 2.

As in *Proales sordida* (Jennings and Lynch, 1928—I), four periods may be distinguished in the life of the individual: (1) The period of embryonic development, from the deposition of the egg to hatching; this requires, in *Lecane inermis*, about one and one-half days. (2) The immature period, one of rapid growth and activity, concluding as a rule on the third day with the deposition of the first egg. (3) The period of fecundity, five or six days during which the eggs one at a time are matured and deposited in regular succession. (4) The post-fecund period, or period of old age, during which the activities of the female gradually cease, structural degeneration sets in and death

ensues, usually on the ninth day, though it may be deferred for varying periods, depending on the conditions.

The amictic female.—The term *amictic* was first applied by Storch (1924) to the commonest type of rotifer female, since it cannot reproduce by amphimixis. The cytological investigations by Whitney, Storch, Nachtwey and others, of several species of rotifers, have shown that the eggs of the amictic female invariably develop by diploid parthenogenesis and produce diploid females, which may be either amictic or mictic.

The amictic females of *Lecane inermis* produce eggs that are ellipsoid in form and measure about 64 μ by 38 μ (Fig. 6). They are covered with a thin membrane surrounded by a gelatinous layer which swells, separates from the egg and disappears before the embryo emerges, about 30 hours after deposition.

The mictic female is capable of reproduction by amphimixis. Its eggs, as shown by the above-mentioned investigators, undergo reduction in the number of chromosomes. The mictic female of *Lecane inermis*, although indistinguishable in appearance from the amictic female, is immediately recognizable by the kinds of eggs it produces.

EXPLANATION OF PLATE

These drawings were reconstructed from many camera lucida sketches, for which a Zeiss microscope was used with oc.15x, obj.8.3, and with oc.15x, (Leitz) obj.3. The magnification of the seven figures is 518 diameters; this magnification was obtained by doubling the average dimensions of sketches of eggs and of living individuals, made with oc.15x, (Leitz) obj.3.

Abbreviations

c, contractile vacuole
d, rudiment of anterior portion of the digestive system
g, gastric glands
ex, excretory (?) granules
i, intestine
m, mastax
p.g., prostate (?) glands
o, ovary
oe, œsophagus
r, retro-cerebral organ
t, toes
te, testis
v.d., vas deferens

FIG. 2. Mature female (mictic or amictic); dorsal view.

FIG. 3. Young female, shortly after hatching; lateral view, showing prominent mastax, gastric glands, œsophagus, intestine and contractile vacuole.

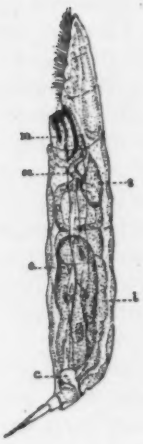
FIG. 4. Male; dorsal view.

FIG. 5. Male; lateral view, for comparison especially with Fig. 3. The digestive system is represented only by an anterior rudiment; the contractile vacuole is absent.

FIG. 6. The (female-producing) egg of the amictic female, several hours after deposition; lateral view. The gelatinous layer has separated from the egg membrane.

FIG. 7. The (male-producing) egg of the mictic female, before the initiation of cleavage.

FIG. 8. The fertilized (female-producing) egg of the mictic female.



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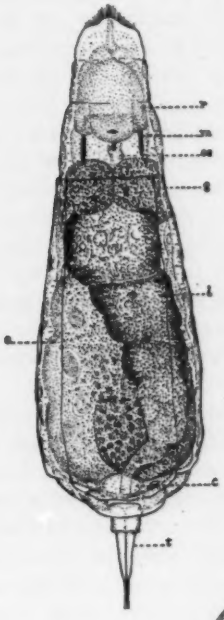

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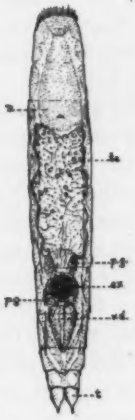

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If unfertilized, the eggs (Fig. 7) resemble the female parthenogenetic eggs, except that they are smaller (44 μ by 32 μ) and produce males.

The fertilized eggs (Fig. 8) are larger (64 μ by 38 μ), covered with a thick, horny shell, and produce amictic females. They are very black when laid, but the color fades to light brown in the course of several days. In this condition they remain for a period varying from a few days to two months. They withstand drying, which does not seem to alter their hatchability. Ordinarily, only 20 per cent to 30 per cent hatch in spring water or in oat infusion. Further investigation of the hatchability of the fertilized eggs is much needed. The course of development of these eggs has not been studied in detail. Apparently, cleavage begins shortly after deposition, but the greater part of embryonic development occurs during the 24 or 48 hours prior to hatching. The embryo emerges through an irregular break in the side of the shell.

The life histories of 108 amictic and 111 unfertilized mictic females were studied and compared in detail. These individuals were characterized by a high degree of genetic uniformity, since they were derived recently by parthenogenesis from a single common ancestor, and since they were derived from young mothers, less than 4 days old. This latter precaution eliminated the possibility of certain intrinsic differences, in fecundity, embryonic mortality and in the duration of certain periods in the life history, which result, in *Proales sordida* (Jennings and Lynch 1928–I, II), from differences in the ages of the parents.

The experimental females were cultivated individually under similar conditions, in small groups, during a period of two months. Uniformity of cultural conditions was obtained as follows. Small mass cultures, of 15 to 25 individuals in three drops of oat infusion, were maintained, the individuals being transferred daily to new fluid. Female-producing parthenogenetic eggs were removed from these cultures at the time of daily transfer, and isolated in depression slides, each one in three drops of oat infusion. The eggs isolated at any one time had thus been produced during the preceding 24 hours. The records of ages begin at the time of isolation, so that they are accurate only to a period of within 24 hours.

The eggs hatched as a rule during the first day after isolation. The young females grew rapidly and began to deposit eggs on the second day. Not until then could the individuals be recognized as mictic or amictic. They were transferred daily to clean slides and twenty-four hour infusion, at which time the number of eggs produced and other details were recorded for each mictic and amictic female.

TABLE I

Lecane inermis. Records of the numbers of eggs produced daily, throughout life, and of the total life duration, for typical contemporaneous amictic and unfertilized mictic females. *n. h.* signifies not hatched (on the first day); *d* signifies that the individual died on the day so marked.

Successive days of life		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total number of eggs	Length of life in days
Number of eggs laid by five amictic females	1	0	3	4	6	4	2	0d										19	7
	2	0	2	5	6	4	3	1d										21	7
	3	n.h.	1	4	5	6	(lost)												
	4	0	3	4	5	3	4	0d										19	7
	5	0	4	5	5	4	2	1	0d									21	8
Number of eggs laid by seven unfertilized mictic females	1	n.h.	1	4	4	4	3	0	0	0	0	0	0	0	0	0	0d	16	16
	2	0	3	4	4	3	0	0	0	0	0	0d						14	11
	3	0	3	4	5	3	0	0d										15	7
	4	0	3	4	4	4	1	0	0	0	0d							16	10
	5	0	2	3	4	4	3	0	0	0	0	0d						16	11
	6	1	4	4	3	2	0	0	0	0d								14	9
	7	1	4	4	4	3	0	0	0	0	0d							16	10

Since the male embryos require 30 hours or more for development, fertilization of the mothers by the sons was impossible, so that the mictic females as well as the amictic females reproduced throughout life only parthenogenetically.

During the two months of cultivation, the temperature varied from 19° to 24° C. Daily variations did not exceed one and one-half degrees. Although the mictic and amictic individuals were not distributed throughout this period in equal numbers, certain striking differences are observable in the life histories of the two kinds of females that cannot be attributed to differences in temperature, since they are exhibited by contemporaneous individuals subjected as nearly as possible to the same conditions of culture. Large contemporaneous cultures of the two types of females were at the time impracticable.

Table I gives the life histories with relation to the number of eggs produced daily, and the length of life, for two small groups of contemporaneous females of the two types. From Table I it is at once apparent that the mictic females (unfertilized) produce fewer eggs than the amictic females, but usually live longer. Further, on the whole, the egg-laying period of the mictic females terminates earlier.

Length of life and distribution of mortality

Table II gives for the individuals of the amictic and mictic populations the length of life in days (after their isolation as eggs); also the biometric constants, and the proportions that lived for the modal number of days, less than the mode and more than the mode.

As Table II shows, the largest number of individuals died, in both populations, on the ninth day, so that this is the modal length of life for both sets. But many of the mictic females (55.9 per cent) lived beyond this age, while fewer of the amictic females (32.4 per cent) did so. The maximum age reached by the amictic females was 15 days, while 13 per cent of the mictic females lived beyond this age, two reaching the ages of 27 and 28 days respectively. The mean length of life of the amictic females is 8.9 ± 0.11 days; of the mictic females, 11.0 ± 0.28 days. There are thus very characteristic and significant differences in the length of life reached by the amictic and mictic females respectively. Further, the duration of life is much more variable for the mictic than for the amictic females. For the amictic females the standard deviation in length of life is 1.72 ± 0.08 days, and the coefficient of variation is 19.28 ± 0.92 per cent, while for the mictic females the standard deviation is 4.37 ± 0.20 and the coefficient of variation 39.41 ± 2.04 per cent.

These differences in length of life are very noticeable in observation

TABLE II

Lecane inermis. Length of life in days (after their isolation as eggs) of amictic and unfertilized mictic females. The figures given in the body of the table are the numbers of individuals of each population that reach the ages in days indicated in the upper line of the table.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Total
Number Amictic Females						7	20	15	31	16	12	5	1	0	1														108
Number Mictic Females					1	3	11	14	20	16	13	8	7	1	3	2	1	1	2	1	2	1	1	1	0	0	1	1	111

	Mean	Standard Deviation	Coefficient of Variability	Mode	Per cent below mode	Per cent at mode	Per cent above mode
Amictic Females	8.90 ± 0.11	1.72 ± 0.08	19.28 ± 0.92	9	38.88	28.70	32.41
Mictic Females	11.10 ± 0.28	4.37 ± 0.20	39.41 ± 2.04	9	26.13	18.02	55.86
Difference	− 2.20 ± 0.30	− 2.65 ± 0.21	− 20.13 ± 2.24	0	12.75	10.68	− 23.45

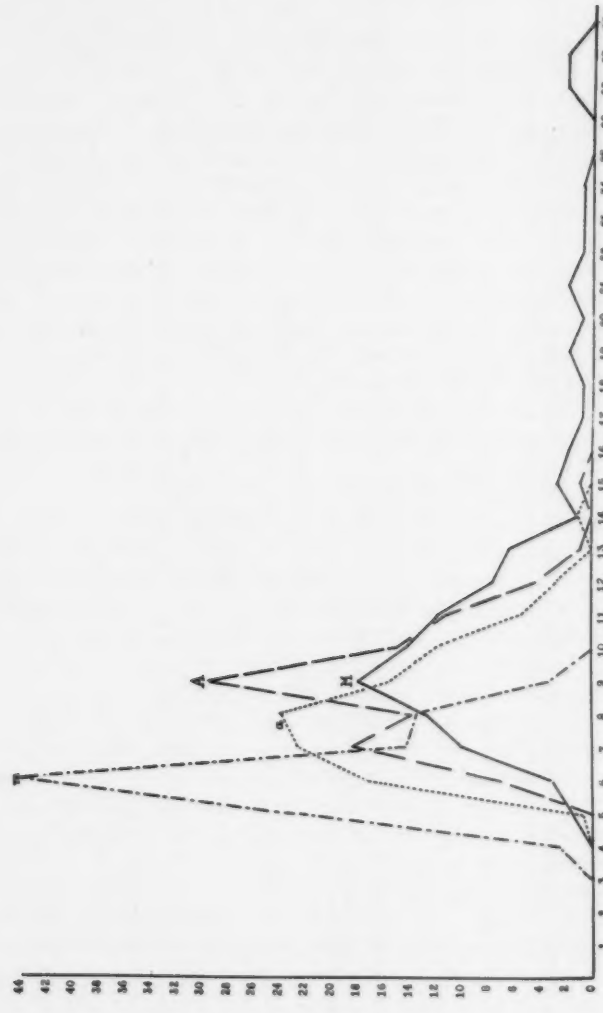

FIG. 9. *Lecane inermis*. Graphs showing the proportions of individuals reaching the different ages, in the amictic population (*A*), and in the population of unfertilized mictic females (*M*); also the ages at which the amictic females (*a*) and the unfertilized mictic females (*m*) cease egg-deposition. Age reckoned in days from time of isolation (within 24 hours after deposition). Horizontal scale, days of life; vertical scale, percentages of each population.

of the living animals. The mictic females cease egg-laying early in life, but most of the individuals continue to swim about actively for several days after, a few of them for many days. In these long-lived individuals physical decline is very gradual. The gastric glands and mastax become blackened and locomotion wanes, typical signs of structural degeneration. But the mictic females may linger on for many days in this condition. The amictic females, on the other hand, frequently show signs of age before the last egg is produced and all are dead within two days thereafter.

The distribution of mortality is represented graphically in Figs. 9 and 10. In Fig. 9, curves *M* and *A* show the proportions of the mictic and amictic populations respectively that reached the different ages. Figure 10 represents mictic and amictic individuals as though contemporaneous, and shows the proportions of each population present at the beginning of each day. Embryonic mortality is negligible; only one egg of the 220 eggs isolated failed to hatch. In this respect early-born mictic and amictic females resemble early-born populations of *Proales sordida* (Jennings and Lynch, 1928—II, Fig. 15). None die during immaturity; all live through the fourth day. All of the amictics and 99 per cent of the mictics survive the fifth day after isolation, that is, four days of fecundity. On every day thereafter, fewer amictic than mictic females are still alive. In both populations the mortality rate is low on the sixth day, higher on the next and reaches a maximum on the ninth day. It thereafter decreases until all the amictics are dead on the fifteenth day and the mictics linger on, two or three dying on successive days until the twenty-eighth day.

To what extent is the difference in longevity of the mictic and amictic females correlated with, or the result of, difference in fecundity? Information regarding this question is obtained from examination of the distribution of fecundity in the two populations, the rate of egg-production, the duration of the post-fecund period and the age at which the production of eggs ceases.

Fecundity

Inspection of Table I, giving the life history records for contemporaneous mictic and amictic females, has shown that the mictic female deposits only 14 to 16 of the small, male-producing eggs; the amictic female deposits 19 to 21 of the larger, female-producing eggs. Table III presents a comparison of the distribution of fecundity in the two populations, with the biometric constants; and Fig. 11 shows the proportions of individuals that produced the different numbers of eggs. The differences in the fecundity of the two types of female are striking. The amictic females produce 13 to 24 eggs per individual; the modal

FIG. 10. *Lecane inermis*. Mortality and survivorship diagrams for the three different types of individuals, the amictic females (*A*), the unfertilized mictic females (*M*) and the males (♂). Horizontal scale, days of life; vertical scale, percentages of the populations surviving on the successive days.

TABLE III

Lecane inermis. Comparison of the fecundity of amictic and unfertilized mictic females. Total number of eggs produced during its life-time by each of the 111 mictic and 108 amictic females.

Number of eggs	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Total
Number amictic females.....														1	0	1	3	3	5	11	18	27	17	16	6	108
Number mictic females.....			1	0	0	0	0	0	0	0	0	2	10	15	29	31	23									111

	Mean	Standard Deviation	Coefficient of Variability
(*a*) Amictic Females (108).....	20.73 ± 0.13	2.06 ± 0.09	9.92 ± 0.46
(*b*) Mictic Females (111).....	14.22 ± 0.11	1.74 ± 0.08	12.22 ± 0.56
(*c*) Mictic Females, without individual that produced 2 eggs (110)....	14.33 ± 0.08	1.29 ± 0.06	9.03 ± 0.41
Differences *a*–*b*.....	6.51 ± 0.17	0.32 ± 0.12	− 2.30 ± 0.73
a–*c*.....	6.40 ± 0.16	0.77 ± 0.11	0.89 ± 0.62

fecundity is 21 and the mean 20.7 ± 0.13. The mictic females produce 11 to 16 eggs (with the exception of one individual which produced only two eggs and died on the fifth day); the modal fecundity is 15, and the mean 14.2 ± 0.11. Thus, the mictic females produce regularly only two-thirds as many parthenogenetic eggs as the amictic females. The fact that the coefficients of variability for both populations are low, 9.9 per cent for the amictics and 12.2 per cent for the mictics, emphasizes the difference in the fecundity of the two types of females.

Excluding the single exceptional mictic female which produced only two eggs, the mean fecundity for the mictic population (110 individuals) is 14.3 ± 0.08, the standard deviation 1.29 ± 0.06, which is less than that of the amictic population by 0.77 ± 0.11, a significant difference, and the coefficient of variability is reduced to 9.03 ± 0.41, which is almost the same as that of the amictic females.

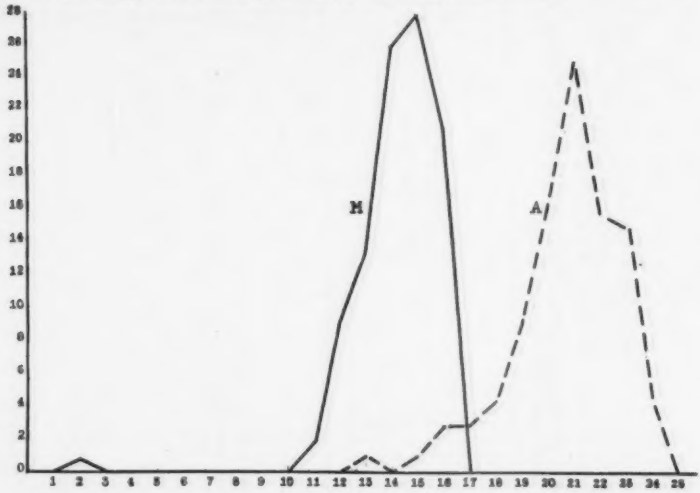

FIG. 11. *Lecane inermis*. Graphs showing the numbers of eggs produced by individuals of the two populations, the amictic females (*A*) and the unfertilized mictic females (*M*). Horizontal scale, numbers of eggs produced; vertical scale, percentages of the populations producing the different numbers of eggs.

The two populations differ further in the fact that a very much greater proportion of the mictic females produced the maximum or nearly the maximum number of eggs. This is clearly observable in Fig. 11. Whereas 48 per cent of the mictic females produced 15 or 16 eggs, only 21 per cent of the amictics produced 23 or 24 eggs. The mode and the mean fecundity more nearly coincide with the maximum in the mictic population.

Duration of the fecund period and rate of egg-production

It will be recalled that the mictic and amictic females mature and deposit the first eggs at about the same time, during the second day after isolation, also that the mictic female deposits but two-thirds as many eggs as the amictic female. If the male-producing parthenogenetic eggs are deposited at the same rate as the female-producing eggs, the fecund period of the mictic female should be only two-thirds as long as that of the amictic female.

TABLE IV

Lecane inermis. Duration of the period of fecundity of amictic and unfertilized mictic females. The figures given in the body of the table indicate the number of individuals in each population that required for the production of their eggs the number of days indicated on the upper line.

Number of days	2	3	4	5	6	7	8	9	10	11	Total
Number of Amictic Females.....			4	30	30	19	14	9	1	1	108
Number of Mictic Females.....	1	0	22	57	26	5					111

	Mean	Mode	Standard Deviation	Coefficient of Variability
(*a*) Amictic Females (108)....	6.42 ± 0.09	5, 6	1.44 ± 0.07	22.45 ± 1.08
(*b*) Mictic Females (111).....	5.10 ± 0.05	5	0.83 ± 0.04	16.22 ± 0.75
(*c*) Mictic Females, without individual that produced 2 eggs and died on 5th day (110).....	5.13 ± 0.05		0.78 ± 0.04	15.13 ± 0.70
Differences *a*–*b*.....	1.32 ± 0.11		0.61 ± 0.08	6.23 ± 1.32
a–*c*.....	1.29 ± 0.10		0.66 ± 0.08	7.32 ± 1.29

In Table IV is given the number of days during which the females of both classes continue to produce eggs. The minimum number of days of the fecund period was the same for both (with the exception of the one individual, which produced only two eggs and died on the fifth day); but the total number of days ranged from 4 to 7 for the mictic females, from 4 to 11 for the amictic females. The mode for the mictics is five days; for the amictics 5 and 6 days are equally common. For 23 per cent of the amictics the period of fecundity exceeds the maximum period for the mictics. The mean for the mictics is 5.10 ± 0.05 days, for the amictics 6.42 ± 0.09 days. The standard deviation is, for the amictics 1.44 ± 0.07, for the mictics 0.83 ± 0.04. The coefficient of variability is, for the amictics 22.45

$\pm$ 1.08, for the mictics, 16.22 $\pm$ 0.75. The differences in the mean, standard deviation and the coefficient of variability of the two populations are statistically significant, regardless of whether the one exceptional mictic female is included in the calculations (Table IV).

The mictic female requires on the average only one and three-tenths days less to produce its eggs, though these are fewer by one-third than the number produced by the amictic female. This means that, although both types of females produce 3 to 5 eggs per day, rarely 6, the mictic female deposits its small (male-producing) eggs, on the average, in less rapid succession than the amictic female deposits its larger (female-producing) eggs. The mictic female deposits, on the average, 3.8 eggs per day, or one every 8.6 hours; the amictic female 3.2 eggs per day, or one every 7.5 hours. These figures represent the quotients of the mean fecundity divided by the mean duration of the fecund period. Additional data regarding the rate of egg-deposition are given later in the paper.

Age at cessation of fecund period and duration of post-fecund period

In Table V is given the duration of the post-fecund period for all individuals of the two populations. Most of the amictic females (59 per cent) died within 24 to 36 hours after deposition of the last egg; 33 per cent died within one day, all died within two days thereafter.

TABLE V

Lecane inermis. Duration of the post-fecund period of amictic and unfertilized mictic females. Those individuals that produced eggs on the day on which they died, or as many as four eggs on the day preceding death are considered as having a post-fecund period of one day or less; if they produced no eggs on the last day, but one to three on the preceding day, they are listed as having a post-fecund period of one to one and one-half days; if they produced no eggs on the last two, three or four days, their post-fecund period is correspondingly two, three or four days, etc.

Number of Days	One day or less	1–1½	2	3	4	5	6	7	8	9	10
Number of Amictic Females.....	36	64	8								
Number of Mictic Females.....	2	15	20	18	17	6	11	2	2	3	3

Number of Days	11	12	13	14	15	16	17	18	19	20	21	22	Total
Number of Amictic Females...													108
Number of Mictic Females....	1	1	3	1	2	0	0	2	0	0	1	1	111

Not so with the mictic females. Only 33 per cent of the entire population died within two days after the cessation of egg-deposition.

Of these, 20 individuals survived for two days, 15 for one and one-half days, only two died within 24 hours after deposition of the last egg. While two days after the cessation of egg-production is the commonest period of death, three or four days are almost equally common; five and six days less frequent. A few individuals survive 7 to 22 days after the cessation of egg-production, there being, however, but one, two or three individuals in each class. Yet 20 per cent of the mictic females lived more than 6 days after the fecund period had ended.

As set forth in the preceding section, the mictic females cease to deposit eggs earlier, on the average, than the amictic females. The post-fecund period of the mictic females is thus extended in this way by about 1.3 days. However, this accounts for only a small fraction of the greater length of life of the mictic females after the cessation of egg-deposition. The mictic females have, on the whole, a much greater ability to survive the period of fecundity than the amictics.

This striking difference is shown for the entire populations in Fig. 9, representing the distribution of ages at the cessation of egg-production in comparison with the distribution of ages at death.

TABLE VI

Lecane inermis. Comparison of the ages at which amictic and unfertilized mictic females cease the deposition of eggs.

Age in Days	4	5	6	7	8	9	10	11	12	13	14	Total
Number of Amictic Females.....	0	1	19	25	26	17	13	6	0	0	1	108
Number of Mictic Females.....	3	24	49	16	15	4						111

Table VI gives for both populations the number of individuals that cease egg-production at the different ages. The fecund period of the amictic females terminates usually on the seventh and eighth days; that of the mictic females usually on the sixth day. As stated in a previous section, all of the amictic females and all but one mictic female live through four days of egg-production. Mortality then begins in both populations and rises to a maximum on the ninth day. Thus, the maximum mortality in the amictic population occurs from one to two days after the maximum number of individuals cease egg-production; in the mictic population, three or four days thereafter. After the ninth day, the distribution of mortality of the amictic females follows the cessation of egg-production within one or two days. The mictic females, however, have all ceased egg-production at the end of the ninth day, and 56 per cent are still alive. These die at a slow rate until the 14th day, the remaining 15 per cent at a fairly uniform rate until the 28th day.

Relation of length of life to fecundity and rate of egg-production

The foregoing data indicate that the difference in the length of life of mictic and amictic females results, in part, from the difference in their ability to survive the fecund period. This has been evidenced by the relative duration, in the two populations, of the fecund and post-fecund periods, and the relative ages at which egg-production ceases.

We may inquire further into the extent of the relationship between length of life and fecundity of mictic and amictic females, by comparing the fecundity, rate of egg-production, and the duration of the post-fecund period, in relatively short-lived, average-lived and long-lived individuals of each population. Those that lived less than 9 days, the mode, are classified arbitrarily as short-lived, those that lived 9 days as average-lived and those that lived more than 9 days as long-lived. The results of this comparison are presented in Tables VII to IX.

TABLE VII

Lecane inermis. Comparison of the relation of life duration to fecundity displayed by amictic and unfertilized mictic females. Individuals that lived less than 9 days, the mode, are called short-lived; those that lived 9 days are called average-lived, and those that lived more than 9 days, long-lived.

		Short-lived	Average-lived	Long-lived		Total
				Total	Those living beyond 15 days	
Amictic Females	Number	42	31	35	0	108
	Mean eggs	19.95	20.45	21.91		
Mictic Females	Number	29	20	62	14	111
	Mean eggs	14.0	14.05	14.2	14.64	

Table VII gives the mean fecundity for these groups. The short-lived amictic females produced, on the average, 19.95 eggs per individual, the average-lived 20.45, the long-lived 21.91. The difference in the mean fecundity of short-lived and long-lived amictic females is 1.96 eggs. The short-lived mictic females produced, on the average, 14.0 eggs per individual, the average-lived 14.05, the long-lived 14.2. The difference in the mean fecundity of short-lived and long-lived mictic females is 0.2 eggs, which is only one tenth of the difference (1.96) found in the corresponding groups of amictic females. The mean fecundity of those mictic females that lived beyond 15 days, and thus survived all the amictic females, is 14.6 eggs; that is, it is

greater than the mean fecundity of short-lived mictic females by only 0.6 eggs. The positive correlation between length of life and the number of eggs produced is appreciably more marked for the amictic females.

TABLE VIII

Lecane inermis. Comparison of the relation of life duration to rate of egg-deposition (number of eggs per day) for amictic and unfertilized mictic females. The "mean rate" of egg-deposition is the average of the rates at which all the members of a particular group deposited their eggs. The rate of egg-deposition of each individual is computed by dividing the total number of eggs produced, by the number of days on which eggs were deposited by that individual.

		Short-lived	Average-lived	Long-lived		Total
				Total	Those living beyond 15 days	
Amictic Females	Number	42	31	35	0	108
	Mean rate	3.88	3.33	2.73		
Mictic Females	Number	29	20	62	14	111
	Mean rate	3.0	2.78	2.78	2.87	

The length of life of the amictic females bears a negative correlation with the rate at which they produce their eggs (Table VIII). The short-lived individuals are found to have deposited their eggs in more rapid succession than individuals that lived 9 days or longer. They deposited, on the average, 3.9 eggs per day, the average-lived 3.3 eggs per day, the longer-lived 2.7 eggs per day. The short-lived mictic females deposited 3 eggs per day, the average-lived 2.8, the long-lived also 2.8 eggs per day. Those in the latter group that lived 16 days or more deposited, on the average, 2.9 eggs per day. The length of life of the mictic females apparently bears little relation to the rate at which their eggs are deposited.

It should be noted also that each group of mictic females is characterized by an average daily egg-production almost the same (within 0.3 eggs) as the group of amictic females that produced their eggs most slowly, namely, the long-lived individuals. The amictic group with the highest rate of egg-deposition (the short-lived individuals) produce, on the average, 0.9 eggs per day more than the corresponding group of mictic females. These facts confirm and supplement data given in a previous section which indicate that the mictic female produces its eggs more slowly than the amictic female.

We find, furthermore (Table IX), in the amictic population, that whereas 25 (60 per cent) of the short-lived individuals died within 24

TABLE IX

Lecane inermis. Comparison of the relation of length of life to the duration of the post-fecund period for amictic and unfertilized mictic females. The figures in the body of the table indicate the number of individuals that survived after the cessation of egg-production for the number of days indicated on the upper line.

Number of Days		1 or less	1 to 1½	2	3	4	5	6	7	8	9–22	Total
Short-lived Females	Amictic	25	16	1								42
	Mictic	2	9	14	4							29
Average-lived Females	Amictic	6	22	3								31
	Mictic		5	2	8	5						20
Long-lived Females	Amictic	5	26	4								35
	Mictic		1	4	6	12	6	11	2	2	18	62
Total number	Amictic											108
	Mictic											111

hours after deposition of the last egg, only 6 (19 per cent) of the average-lived and 5 (14 per cent) of the long-lived died within this period. Twenty-two (71 per cent) of the average-lived and 26 (74 per cent) of the long-lived females survived for 24 to 36 hours after deposition of the last egg. Only 8 individuals (7 per cent of the entire population) survived as long as two days after the cessation of egg-production.

However, all of the mictic females, with the exception of two short-lived individuals, lived more than 24 hours after the cessation of egg-production. Most of the short-lived mictic females survived one and one-half or two days. The average-lived survived usually three days, though one and one-half and three days were almost equally common. The long-lived individuals, which constitute 56 per cent of the population, survived one and one-half to 22 days after the cessation of egg-production, commonly 4 to 6 days. Only 10 per cent survived less than 4 days; 20 per cent survived longer than 6 days.

In short, the amictic females that lived for less than 9 days produced their eggs, on the average, in more rapid succession than those that lived longer; they also produced fewer eggs and died usually within 24 hours after deposition of the last egg. These facts suggest that rapid rate of egg-production exhausts the short-lived amictic females, rendering them incapable of completing the transformation of their

germ cells into eggs, thus reducing their fecundity. So strenuous is the fecund period that none is able to survive more than two days after its cessation.

In the mictic population, as in the amictic population, the longest-lived individuals are those that have, on the average, produced their eggs more slowly, have produced a larger number of eggs and have survived more than a day after the deposition of the last egg. However, the length of life of the mictic female is correlated in less degree than that of the amictic female with the number of eggs produced and the rate at which they are produced. The short-lived mictic females usually survive as long after the cessation of egg-deposition as the longest-lived amictic females; they very infrequently show signs of physical exhaustion before the completion of egg-production. We may conclude, then, that the greater longevity of the mictic females, which involves a greater ability to survive the fecund period, results, in part at least, from the fact that for them the process of egg-production is less strenuous. This is to be expected in view of the fact that they produce fewer, smaller eggs at a less rapid rate than the amictic females.

General and comparative

Under similar conditions of cultivation amictic and unfertilized mictic females of *Lecane inermis* differ markedly in length of life, fecundity, rate of egg-production, duration of the fecund and post-fecund periods and in the degree of correlation between fecundity and length of life. How are these differences between the two types of females produced?

In their investigation of the diversities that arise within a clone of (amictic) females of the parthenogenetic rotifer, *Proales sordida*, Jennings and Lynch (1928) concluded that differences in length of life, among those individuals that hatch, are not an expression of intrinsic diversity. They result, rather, from the accidents of life, the interaction of the rhythmic processes of digestion and reproduction, combined with disturbances in these processes imposed by the daily transfer of the individuals to clean slides.

In *Lecane inermis*, the difference in the length of life of the mictic and amictic female results largely from the difference in the severity of the process of egg-production, hence in their ability to survive the fecund period. Both mictic and amictic females lived most commonly nine days, but many more mictic than amictic females lived beyond this age, presumably because they produce fewer, smaller eggs than the amictic female, at a slower rate, and cease egg-deposition at an

earlier age. Thus, in *Lecane inermis*, differences in length of life are probably to be considered of an intrinsic nature only in so far as they result from intrinsic differences in fecundity.

The mictic females of *Lecane inermis* resemble the females of *Proales sordida* in that some of them are able to live for many days after the cessation of egg-production. The amictic females of *Lecane inermis*, however, never survive more than 3 or 4 days thereafter. Both mictic and amictic females differ from *Proales sordida* in the degree of correlation between length of life and fecundity. For individuals of *Proales sordida* that live entirely through the period of fecundity (24 hours after deposition of the last egg) there is no correlation between length of life and the number of eggs produced; longer-lived individuals have, on the whole, produced neither fewer nor more offspring than the average (Jennings and Lynch, 1928—II, p. 371). In *Lecane inermis*, on the other hand, among those individuals that lived entirely through the fecund period, the longer-lived amictic females and those mictic females that lived for more than 15 days have a somewhat greater average fecundity than those that lived a shorter time.

What is the origin of the difference in fecundity which is, in *Lecane inermis*, largely responsible for the difference in longevity of the mictic and amictic female? In *Proales sordida*, intrinsic diversities in fecundity arise among the individuals of a clone in correlation with the size of the eggs from which they have hatched. Smaller (usually early-born) eggs produce less fecund individuals than larger (later-born) eggs. In order to eliminate diversities that might arise in this way, only early-born individuals were used in the present investigation. Although measurements have not been made, there is no observable difference in the size of the parthenogenetic eggs that produce the two different kinds of females of *Lecane inermis*. In the viviparous species *Asplanchna intermedia*, on the other hand, the mictic female is apparently derived from a smaller embryo and is less fecund than the amictic female (Tauson, 1925, p. 144).

As Jennings and Lynch point out (with respect to *Proales sordida*), differences in fecundity must proceed either from differences in the ability of the individuals to transform their germ cells into eggs under the conditions of cultivation employed, or from differences in the original number of their germ cells. There is (as they observe) evidence that the number of germ cells, as well as of the other cells of the body of rotifers, is fixed during the embryonic period. Cell mitoses are not observed later in life. Nachtwey (1925) has shown that the rotifer *Asplanchna priodonta* has usually 32 germ cells,

derived by five cell cleavages from the single primitive germ cell. In view of this evidence, Jennings and Lynch (1928—I) have suggested that differences in the fecundity of the (amictic) females of *Proales sordida* that are derived from smaller eggs and those derived from larger eggs may result from differences in the original number of germ cells. Late-born individuals may have usually 32 germ cells, early-born individuals a smaller number, resulting from failure of a portion of the fourth cell generation to undergo another division.

In *Lecane inermis* the observed difference in the fecundity of the two types of female would result if the primitive germ cell of the mictic female undergoes regularly only four successive divisions, producing 16 oöcytes; and if, in the amictic female, one-half of the fourth cell generation regularly proceeds to a fifth cleavage, thus producing 24 germ cells. Cytological investigation would, of course, be required in order to determine whether there exists a difference in the original number of germ cells.

It is possible that, under conditions other than those of this investigation, the mictic female might be able to bring to maturity as many germ cells as the amictic female. It should be noted, however, that the differences in the fecundity and length of life of the two types of females have been observed during cultivation under widely different conditions; namely, in oat infusion and in Benecke solution at temperatures ranging from 18° to 24° C.

The observations of Whitney (1907) regarding the fecundity of mictic and amictic females of *Hydatina senta* are of interest here. At 20° to 22° C. both types of females produce about the same number of eggs; at 24°–26° C. the mictic female produces twice as many eggs as the amictic; at 26°–29° C. the mictic female produces four times as many eggs as the amictic female. Thus, in *Hydatina senta*, differences in the fecundity of the two types of female result observably from differences in response to the environment. The mictic female lives at all these temperatures slightly longer than the amictic female (Wesenberg-Lund, 1929). The relative longevity of the amictic and mictic female of *Hydatina senta* is not correlated with the relative fecundity, as in *Lecane inermis*.

For other species, information regarding the relative length of life, fecundity and rate of egg-production of the mictic and amictic female is not extensive.

The life history records of three mictic females of *Euchlanis triquetra* (Lehmensick, 1926) indicate that they produce the same number of eggs as the amictic female (24) and live about the same length of time, 21 days. But the mictic female produces its eggs

more rapidly and thus lives longer after the cessation of egg-production.

The mictic female of *Asplanchna intermedia* (Tauson, 1925) produces fewer eggs, as mentioned above, and lives a shorter length of time than the amictic female. The mictic female matures on the first day, produces 10 to 12 eggs on the second day, which hatch on the third day; the mother dies after the hatching of the last male. The amictic female lives 5 or 6 days, produces daily no more than 4 eggs (probably 16 to 20 in all). The mictic female produces its eggs appreciably more rapidly.

In *Pterodina elliptica* (Luntz, 1926), the mictic female produces regularly six or seven eggs, the amictic female only five. There is no available information regarding the length of life. Physiological diversity is manifested by the inability of the mictic female to withstand certain conditions of osmotic pressure and pH in which the amictic female is perfectly normal.

Wesenberg-Lund states (1930, p. 31) that "if not fertilized, investigations hitherto carried out seem to show that the number of eggs laid by the two sorts of females is almost the same, but that those of the mictic female are laid in a much shorter time (Lehmensick, 1926, *Euchlanis triquetra*)."

We find, however, that the mictic females of some species, *e.g.*, *Asplanchna intermedia* and *Lecane inermis*, are regularly less fecund than the amictic females, whereas those of some other species, *e.g.*, *Pterodina elliptica* and *Hydatina senta* are, usually, more fecund. With regard to the relative rates at which the eggs are deposited by the two sorts of females, *Lecane inermis* is exceptional; the mictic female produces its eggs less rapidly than the amictic female.

Wesenberg-Lund (1930) presents a comparison of the mictic and amictic females on the basis of his extensive investigations carried out largely in nature, but supplemented by laboratory observations. The author stresses the physiological and biological differences between the mictic and amictic female and gives some additional data regarding their relative fecundity, rate of egg-production and length of life. In *Asplanchna sieboldi* the mictic females mature and begin to deposit eggs earlier than the amictic females. In isolation cultures they produced 14 to 16 eggs and lived 10 to 12 days. Amictic females, in mass cultures, lived only 8 days and produced 8 to 10 eggs. The author is inclined to believe (p. 156) that in nature the amictic females live longer than the mictics, especially at low temperatures. Concerning *A. brightwelli* he "conjectures that at the same temperatures the amictic females live some days longer than the mictic ones;

produce more young ones; and to a somewhat higher degree are able to accommodate duration of life and time of production of young ones to temperature" (p. 149). Regarding the rate of production of the parthenogenetic eggs, Wesenberg-Lund states (p. 210) that, in several species, "the mictic female is able to subdivide the yolk mass into small amounts, and produce about 12 eggs simultaneously, whereas the amictic females produce the eggs successively." Further evidence of physiological diversity is presented by certain parasitic species in which the amictic females are usually free-living, the mictic females always parasitic. Peculiarities in behavior exhibited by mictic females of some species after fertilization indicate that they are structurally as well as physiologically diverse.

In summary, the evidence at hand is sufficient to demonstrate that in different species the relative fecundity, length of life and rate of egg-production of the mictic and amictic female vary greatly. In every species which has been studied physiological differences between the two types of female are demonstrable; but investigations have not proceeded sufficiently far to admit of general conclusions regarding the nature of the fundamental diversity that distinguishes the mictic from the amictic female.

Fertilized Mictic Females

In the foregoing only unfertilized mictic females have been considered. The mictic female of *Lecane inermis*, like that of *Pterodina elliptica* and *Brachionus bakeri* (Luntz, 1926, 1929), and *Asplanchna sieboldi* (Wesenberg-Lund, 1930, p. 155), may be fertilized during its immature period or after having deposited parthenogenetic eggs. In some species the mictic female can be fertilized only when very young (*Euchlanis triquetra*, Lehmensick, 1926).

The mictic females of *Lecane inermis* resemble those of most species in that they may be fertilized by their own sons. The females of *Pterodina elliptica* are exceptional in that they are incapable of being fertilized by the male progeny of the same grandmother. In *Lecane inermis* cloacal copulation occurs. The toes of both male and female are bent ventrally, almost at right angles to the axis of the bodies, which are extended in opposite directions. The female continues to swim about, pulling the male along with her. Copulation lasts for at least five to ten minutes. The cloacal method of copulation is uncommon in rotifers, according to Wesenberg-Lund (1929). The males of most species attach themselves to the body of the female and in some manner pierce the body wall and deposit sperm directly in the body cavity.

As noted by all investigators of the bisexual rotifers, fertilization produces a visible effect upon the vitellarium. This organ becomes black during the growth of the fertilized egg. This, it is generally believed, results from the deposition of fat and the production of larger, darker yolk granules. In the course of 24 to 36 hours a fertilized egg matures and is deposited. The vitellarium is then quite colorless. The production of yolk and the deposition of a fertilized egg may be repeated four or five times.

Information regarding the fecundity and length of life of fertilized mictic females of this species is derived from the life history records of 18 individuals fertilized during immaturity and 5 individuals fertilized by their own sons. The individuals fertilized before maturity produced only fertilized eggs, the total number not exceeding five. They lived usually 4 or 5 days after the cessation of egg-production; the total length of life ranged from 10 to 25 days.

The life history records of the five females fertilized by their own sons at various times during maturity are given in Table X. These individuals produced 5 to 9 parthenogenetic eggs before being fertilized. They produced thereafter one to 4 fertilized eggs. They are typical in their ability to survive the fecund period. The total length of life ranged from 13 to 18 days.

Mictic females of this species have not been observed to deposit parthenogenetic eggs after once having produced fertilized eggs. This is not the case in *Hydatina senta* (Shull, 1910) and in several other species. Lehmensick (1926) suggested that fertilized mictic females return to the production of parthenogenetic eggs because all of their germ cells were not fertilized at the first mating, and Wesenberg-Lund has observed that after a second mating the production of fertilized eggs may be resumed (1930—II, p. 154). The failure of fertilized mictic females of *Lecane inermis* to produce parthenogenetic eggs probably indicates that more of their germ cells were fertilized than they were capable of bringing to maturity; for, frequently, after the cessation of egg-deposition the vitellaria contained dark yolk granules and fertilized eggs were present in various stages of growth.

Fertilization commonly reduces the fecundity of the mictic female. *Lecane inermis*, which produces, when fertilized, only 5 to 10 eggs rather than 16, is not exceptional in this respect. The length of life is, in this species, apparently not affected by fertilization.

The Males

The males of this species (Figs. 4 and 5) are strikingly different in appearance from the females, and, like most male rotifers, are to

TABLE X

Lecane inermis. Records of the numbers of male-producing and fertilized eggs produced daily, throughout life, and of the total life duration of five mictic females, fertilized at various times during the fecund period. The numbers given in the body of the table are the numbers of eggs deposited, on the days indicated on the upper line, by each of the five individuals. The designation (♂) below the numbers indicates male-producing eggs; numbers not so marked indicate fertilized eggs.

Individuals	Days of Life																		Fecundity			Life in days
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Male eggs	Fertilized eggs	Total	
1	0	5 ♂	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0d	5	2	7	18
2	0	0	2 ♂	3 ♂	2 ♂	0	1	1	1	1	0	0	0d						7	4	11	13
3	0	0	3 ♂	3 ♂	2 ♂	1 ♂	1	0	0	0	0	0	0	0	0	0	0	0d	9	1	10	18
4	0	1 ♂	3 ♂	3 ♂	1 ♂	1	1	0	0	0	0	0	0	0	0	0	0d		8	2	10	17
5	0	1 ♂	2 ♂	2 ♂	1 ♂	1	1	1	0	0	0	0	0	0d					6	3	9	14

some extent degenerate in structure. The females are somewhat flask-shaped, flattened dorso-ventrally, and become pigmented as they age. The males, on the other hand, are nearly cylindrical, transparent, and are somewhat shorter and about one-half the width of the newly-hatched female. The toes of the female are long and slim, those of the male are short and thick. The lorica of the male, especially in the posterior region, shows a greater degree of segmentation than that of the female, and the body is thus more flexible. One of the most striking peculiarities of the male rotifer is a black, disk-shaped body. It consists of a vesicle containing large, dark granules, which are supposed by Leydig to be excretory in nature (Wesenberg-Lund, 1929, p. 327). This structure is located dorsally in the posterior region of the body.

The females more than double their size through growth (compare Figs. 2 and 3); the males do not grow. This lack of the power of growth is characteristic of the males of most species of rotifers and is associated with the absence of a functional digestive system. In *Lecane inermis*, no traces of the mastax and digestive glands, so prominent in the female, have been observed in the male. Apparently, only the rudimentary anterior portion of the digestive tube persists, and this serves, as in other species (Wesenberg-Lund, 1923, 1929), as a suspensor ligament for the testis (compare Figs. 3 and 5).

The excretory system shows some structural reduction in the absence of the contractile vacuole.

The reproductive system is well-developed. The testis is large and fills most of the body cavity. The vas deferens is also conspicuous. Two large bodies lying ventral to the testis and vas deferens may be the so-called prostate glands, two or four of which are usually present in the male rotifers. The structure and method of functioning of the copulatory organ has not been observed.

A prominent retro-cerebral organ and eye-spot are present, as in the female. The activity and flexibility of the male indicate well-developed nervous system and musculature.

The males hatch from the small eggs of the mictic female after a developmental period somewhat exceeding in duration that of the females. Four male parthenogenetic eggs, allowed to develop in standard oat infusion at 23° C., required two hours longer to develop than four female parthenogenetic eggs laid at the same time and subjected to identical conditions during development. The average time required by the male eggs was 31 hours 16 minutes, by the female eggs 29 hours 45 minutes, a difference of one hour and 31 minutes. In a suspension of *Chlorella* and *B. proteus* in Benecke solution of

0.07 per cent concentration, at 22°–24° C., the male embryos require on the average four hours longer for hatching than the female embryos. The duration of the embryonic periods of male and female parthenogenetic eggs deposited by young parents and allowed to develop at the same time are given in Table XI.

TABLE XI

Lecane inermis. Comparison of the duration of the embryonic periods of contemporaneous male and female parthenogenetic eggs, deposited by young mothers, and allowed to develop at 22° to 24° C. The eggs were isolated in pairs on hollow-ground slides, usually a single male and female egg in each depression, in a suspension of *Chlorella vulgaris* and *B. proteus* in Benecke solution of 0.07 per cent concentration.

Number of hours after deposition	Number of contemporaneous eggs hatched	
	Female	Male
30	18	0
32	6	0
34	1	7
36	1	10
38	0	9
39—or longer	0	6
	Total 26	32

Male rotifers have been reported to be sexually mature at the time of hatching or shortly thereafter. This is true also of the males of *Lecane inermis.* During the first few days they are extremely active. They copulate indiscriminately with fertilized or unfertilized mictic females, or with the amictic females. This seems to be generally characteristic of rotifer males. The duration of sexual activity has not been investigated.

The males that hatch live usually 4 to 6 days. They are very active. They swim dorsal side up, commonly in a less direct path, and rotate less than do the females. The males of this species are also typical in the lack of the power of growth. On the fourth or fifth day, activity begins to wane until locomotion ceases entirely. The organism lies on its side in a semi-contracted condition, the anterior portion bent ventrally. This condition culminates in death usually within 24 hours.

For investigation of the length of life and rate of mortality, male

eggs were isolated within 24 hours after deposition, from small mass cultures during a period of three weeks. These individuals, as well as the mictic and amictic populations previously described, belonged to the same pure line (descendants by parthenogenesis of a single stem mother). Of the 110 eggs isolated, 6 individuals that hatched were lost in transferring, and 22 (20 per cent) failed to hatch. Nine of those that failed to hatch were discarded before it was discovered that in many cases the embryos developed completely and were very active within the shell. Only one of the remaining 13 failed to develop. The other 12 individuals lived and were active the usual length of time within the shell; two lived longer than any of those that hatched.

TABLE XII

Lecane inermis. Length of life of 95 males, including 13 which developed but failed to hatch. The length of life is reckoned from the time of isolation of the eggs (within 24 hours after deposition). For comparison of the males with the females, the differences in the biometric constants of the different populations are given at the end of the table. The biometric constants for the female populations are given in Table II.

Number of days	1	2	3	4	5	6	7	8	Total
Those that hatched	0	1	1	4	20	50	6	0	82
Those that developed but failed to hatch	1	0	0	0	1	7	2	2	13

	Mean	Standard Deviation	Coeffieient of Variability
Total studied (95)	5.69 ± 0.07	1.10 ± 0.05	17.48 ± 0.88
Total hatched (82)	5.65 ± 0.06	0.83 ± 0.04	14.73 ± 0.79

Difference between Males and Females			
Amictics (108)—Males (95)	3.21 ± 0.13	0.72 ± 0.09	1.80 ± 1.27
Mictics (111)—Males (95)	5.41 ± 0.29	3.37 ± 0.21	21.93 ± 2.22

Table XII shows the life duration of those males, hatched and not hatched (95 individuals), for which records are complete. They lived from one to 8 days. Sixty per cent of the population died on the sixth day, which was the modal length of life. Twenty-two per cent died on the fifth day and only a very few on any other day. The mean length of life of those that hatched is 5.65 days; including those that

failed to hatch the mean is 5.69 days. The males live three days less than the amictic females, five and one-half days less than the mictic females. The standard deviation is significantly lower than that of both types of females; the coefficient of variability is only slightly less than that of the amictic females, but less than that of the mictic females by 22 per cent.

In Fig. 10, the rate of mortality of male and female populations is compared. Those that developed but failed to hatch are included in the graph. The rate of mortality increases very gradually until the fourth day, at the end of which seven and one-half per cent of the males, but no females are dead. During the fifth and the sixth days, 88 per cent of the male population die, while mortality is just beginning in the female population. All of the males are dead one day before mortality reaches its height in the female population.

The male and female populations differ in hatchability, in the mode and mean length of life, and in the standard deviation; but in the variability of life duration the males and the amictic females do not differ appreciably.

Is the difference in hatchability of the male-producing and female-producing eggs due to an inherent difference in fertility of the mictic and amictic females, or to differences in the ages of the parents or to the conditions of cultivation? The males were, for the most part (77 per cent), derived from females under four days old; all the females studied were deposited by individuals less than four days old. Most of the male-producing eggs that failed to hatch were derived from parents more than four days old, but six were early-born. Thus, 7 per cent of the early-born males and only 0.5 per cent (one in 220) of the early-born females failed to hatch. The female embryos that fail to hatch never live more than one or two days. Since the female and male populations were not cultivated at the same time, however, the failure of male embryos to hatch may have resulted from some environmental difference, and cannot be regarded as conclusive evidence that mictic females produce parthenogenetic eggs that are regularly less fertile than those from amictic females. Information regarding the relative fertility of the parthenogenetic eggs from mictic and amictic females is not available in the literature, in so far as I am aware.

The uniformly short length of life of the males in comparison with that of the females has been observed in other lines investigated on a smaller scale, in which, however, both sexes were cultivated at the same time.

The males of *Lecane inermis* live longer than the males of most

other species hitherto described (Wesenberg-Lund, 1930, p. 158). The large males of the viviparous *Asplanchna* species live four or five days. The moderate reduction in length of life, in comparison with that of the females, is, in *Lecane inermis*, in accord with the moderate degree of structural degeneracy. Differences in the degree of structural degeneracy of the males of different species may bear some correlation with differences in the degree of reduction in the size of the male-producing parthenogenetic eggs and in the rate at which the eggs mature and are deposited, two factors which Wesenberg-Lund (1930) considers to be largely responsible for the structural degeneracy of the male rotifers. Evidence in support of this view is derived from a comparison of the males of *Lecane inermis* with those of the plankton rotifers. The former, which are characterized by a moderate degree of structural degeneracy and of reduction in length of life, are derived from eggs which are appreciably smaller than the female-producing eggs, but which are deposited less rapidly, and require a longer time for development. The males of the plankton rotifers, on the other hand, are derived from very small eggs, a large number of which, according to Wesenberg-Lund, are produced almost simultaneously, and develop rapidly. These males are small, consist of little more than reproductive system and live (in some cases) only a few hours. It should be borne in mind, however, that the tendency toward structural degeneracy and reduced vigor of the male rotifers may result from the haploid condition of their chromosomes (Morgan, 1926, Chapter X). The knowledge regarding these matters is at present very meager.

Summary

This paper is the first contribution of a series dealing with the life cycle of the bisexual rotifer *Lecane inermis* Bryce. The three types of individuals, the mictic females, the amictic females and the males, and the eggs from which they are derived, are described and their life histories are compared statistically. The individuals studied are intrinsically uniform members of the same genetic stock, cultivated under uniform environmental conditions, although they were not all contemporaneous. The comparison is based upon about 100 representatives of each type of individual.

The three types of individuals are characteristically different in length of life. The mean length of life is, for the amictic females, 8.9 ± 0.11 days, for the mictic females, 11.1 ± 0.28 days, for the males, 5.7 ± 0.07 days. This difference is apparent in their life curves. Embryonic mortality is negligible in all three populations. Mortality increases gradually in the male population until the fourth

day, then increases suddenly, 83 per cent of the population dying on the fourth to the sixth days. All are dead by the eighth day. All the females survive through the fourth day; thereafter the rate of mortality increases more rapidly in the amictic population. More than half of the mictic females survive the modal life duration, which is the same for both kinds; 12 per cent to 15 per cent survive the longest-lived amictic female and die off very gradually until the twenty-eighth day. The short life duration of the males is undoubtedly correlated with the structural degeneracy, especially of their digestive and excretory systems.

The longer life of the mictic females, as compared with the amictic females, results, in part at least, from the fact that egg-production is for them a less strenuous process. This is evidenced by the following facts. (1) Their male-producing eggs are smaller. (2) The mictic female produces regularly only two-thirds as many eggs as the amictic female. The maximum number is, for the mictic female, 16, exceptionally 17, for the amictic female 24. The mean number of eggs per individual is, for the mictic female 14.2 ± 0.11, for the amictic female, 20.7 ± 0.13. A higher proportion of mictic females produce their maximum number of eggs. The difference between the mode and the maximum is, for the mictics, only one, for the amictics, three. (3) The mean duration of the period of fecundity of the amictic female is 6.4 ± 0.09 days, with a standard deviation of 1.44 ± 0.07 and a coefficient of variability of 22.5 per cent; the mean for the mictic female is 5.1 ± 0.05 days, the standard deviation 0.83 ± 0.04, the coefficient of variability 16.2 ± 0.75 per cent. (4) During this time the mictic female deposits, on the average, one egg every 8.6 hours, the amictic female deposits one egg every 7.5 hours. (5) The amictic females die usually within 24 to 36 hours after deposition of the last egg; 19 per cent of the mictics lived more than six days thereafter. In summary, the mictic female produces in less rapid succession only two-thirds as many eggs as the amictic female, requires for the production of her eggs 1.3 days less than the amictic female and usually lives longer after the cessation of egg-production.

A comparison was made, for the mictic and amictic females, of the degree of correlation between length of life and their fecundity, rate of egg-production and ability to survive the fecund period. Short-lived amictic females are found to have produced fewer eggs than long-lived individuals, in more rapid succession, and to have died within a few hours after the deposition of the last egg, apparently exhausted by the severity of the process of egg-production. The length of life of the mictic female is correlated only in slight degree

with the number of eggs produced and the rate at which they are produced.

The mictic female of this species may be fertilized during immaturity or after having produced parthenogenetic eggs. The fecundity of the mictic female is reduced by fertilization, the total number of eggs per individual, in 18 cases studied, not exceeding ten. The maximum number of fertilized eggs produced by a single individual was five. The length of life of the mictic female is not appreciably altered by the production of fertilized eggs.

The differences cited above are evidence of fundamental physiological diversity between mictic and amictic females and males of *Lecane inermis*.

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PERMEABILITY AND FATIGUE IN MUSCLE AND ITS BEARING ON THE PROBLEM OF ION ANTAGONISM

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It has been shown by numerous investigators that stimulation of different tissues leads to an increase in permeability which is independent of the particular structure and function.[2] In the case of muscle, Weiss showed that even subminimal stimuli lead to an increase in permeability which was measured by an increase in the output of phosphoric acid by the muscle. When effective stimuli were applied, a close relationship between the intensity of fatigue and the increase in permeability was observed (Embden and Adler, 1922). Furthermore, there was a parallelism between the reversibility of fatigue and that of the changes in permeability. When the muscle recovered from fatigue and the original contractility was again obtained, the permeability was also normal, *i.e.*, no phosphoric acid was given off to the medium in which the muscle was suspended. But if no recovery took place, the output of phosphoric acid remained the same or even increased. These experiments prove that the physico-chemical changes in permeability play an important part in the fatigue problem. It seemed probable that fatigue might be delayed if it were possible to prevent or to diminish the increase in permeability which ordinarily accompanies fatigue. The experiments described in this paper show the correctness of the supposition. Fatigue can be delayed in a medium by which the permeability of the muscle is decreased. The efficiency of different ions in that respect and the general importance of these facts for the problem of ion antagonism will be discussed in this paper.

METHOD

Several hundred experiments on the sartorius muscle of *Rana esculenta* were performed from March 1929 to October 1930. The muscles, prepared in the usual manner, were immersed in an isotonic salt solution and suspended between two platinum electrodes connected with an isotonic lever which magnified the contractions seven times.

[1] Awarded the A. Cressy Morrison Prize in Experimental Biology for 1930 by the New York Academy of Sciences.

Aided by a grant from the Research Fund of the University of Oregon.

[2] Compare Gellhorn, 1929, pp. 166–197.

In the first group of experiments the platinum electrodes were carefully covered with lacquer (except on a point-like spot where the platinum was in contact with the muscle). The muscles were stimulated with rhythmical condenser discharges (apparatus of Scheminsky) 40–60 times per minute so that a decrease in the height of contraction occurred. The two sartorii of the same frog under the same conditions showed an identical course of fatigue (Gellhorn, 1930). This made it possible to study the influence of different ions. The solutions were made up of NaCl, KCl and $CaCl_2$ solutions of $\triangle = 0.39°$. In order to hold the conductivity of the liquid constant and avoid any possible effect on the strength of the stimulus, a part of the sodium chloride solution was replaced by an equal volume of isotonic $CaCl_2$ solution, and thus the effect of different $CaCl_2$ concentrations was studied.

In a second group of experiments another method was used. The sartorii were not stimulated in a liquid but in a moist chamber. The first series of stimuli was applied for five minutes and the muscle was then allowed to recover in an aërated salt solution. Afterward a second series of stimuli was applied until the muscle ceased to respond. The first series of stimuli was designed to show whether both sartorii had the same irritability and fatigability. Pairs of muscles which behaved alike in the first series of stimulation were the only ones considered as material for the determination of the influence of different salt solutions. The effect of these solutions was apparent in the course of fatigue during the second series of contractions. The advantage of this second method consists in the independence of the strength of the stimulus from the conductivity of the solution and furthermore in the exact proof of the identical behavior of the muscles before they were immersed in the salt solutions. In these experiments condenser discharges obtained from the apparatus of Scheminsky (1930) in its improved form were used. Maximal stimuli were given 90 times per minute. Between the first and the second series of stimulations the muscle remained unstimulated for nine minutes in the salt solutions. The experiments of the second group were performed with curarized and uncurarized muscles. The effect of Ca, Sr, Ba, and Mg was studied in reference to the fatigue of muscle.[3]

Experiments Performed with the First Method

Figure 1 shows the course of the height of contractions of the sartorii stimulated 40 times per minute for twelve minutes. One muscle was in NaCl + KCl + $CaCl_2$ solution in which these salts had

[3] After preparation the muscles remained in aërated Ringer's solution for 45–60 minutes before the experiment was begun.

the same concentration as in Ringer's solution, while the other muscle was bathed in NaCl + KCl + $CaCl_2$ which contained eight times as much $CaCl_2$ as the first one. It is apparent that, in spite of the identical height of the contractions at the beginning of the experiment, both

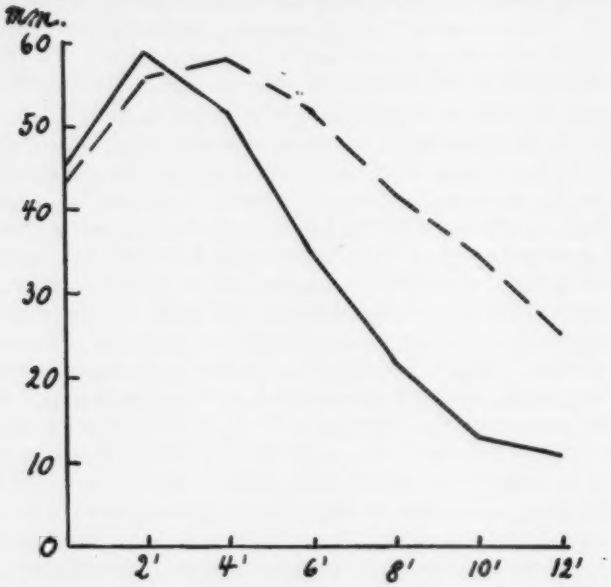

FIG. 1. The course of fatigue in a pair of sartorii stimulated in 0.111 M NaCl + 10.2 M × 10^{-4} KCl + 8.1 M × 10^{-4} $CaCl_2$ (——) and in 0.103 M NaCl + 10.2 M × 10^{-4} KCl + 64.4 M × 10^{-4} $CaCl_2$ (– – –).
Ordinate: height of contraction; Abscissa: time in minutes.
Maximal condenser discharges; frequency 40 per minute.

show the "treppe" in the same amount:—the muscle immersed in a solution with a high $CaCl_2$ concentration showed a lesser fatigue than the control muscle. In other words *an excess of $CaCl_2$ delays fatigue.* This fact, which was the basis for further experimentation, is illustrated by Fig. 2, which reproduces a series of experiments performed with six pairs of sartorii. The control muscle was always stimulated in 0.111M NaCl + 0.00102M KCl + 0.00081M $CaCl_2$, the other muscle in 0.00486M or 0.00972M $CaCl_2$, the KCl concentrations being identical in both cases and the NaCl slightly less in order to keep constant the osmotic pressure and conductivity of the solution. The contractions were registered at intervals of two minutes for a period of twelve minutes and the height of contraction of the muscle stimulated in the

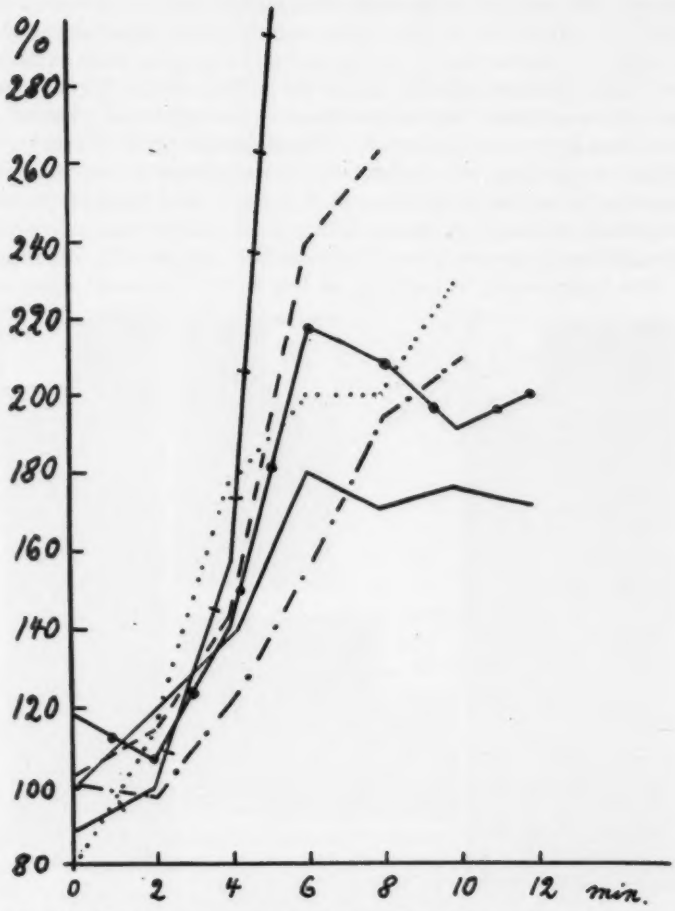

FIG. 2. The influence of different $CaCl_2$ concentrations on the course of fatigue in sartorius muscle. The height of contraction of the control muscle (stimulated in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 8.1 M $\times 10^{-4}$ $CaCl_2$) is taken as 100 and the percentage of the height of contraction of the muscle placed in a solution with higher $CaCl_2$ concentration is calculated.

0.106 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 48.6 M $\times 10^{-4}$ $CaCl_2$.

0.0996 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 97.2 M $\times 10^{-4}$ $CaCl_2$.

higher $CaCl_2$ concentration was calculated as the percentage of the height of contraction of the control muscle. The figure shows that the height of contraction in the beginning of the experiment exhibited very slight variations if any, but in the further course of the experiment the deviations became progressively stronger and reached in most cases 100 per cent or more. This illustrates again the delay of fatigue in solutions with higher $CaCl_2$ concentrations and shows at the same time that the increase of $CaCl_2$ to twelve times its normal concentration (*i.e.*, the concentration in Ringer's solution) is still favorable for the preservation of contractility and the delay of fatigue.

The fundamental importance of this fact is apparent when the

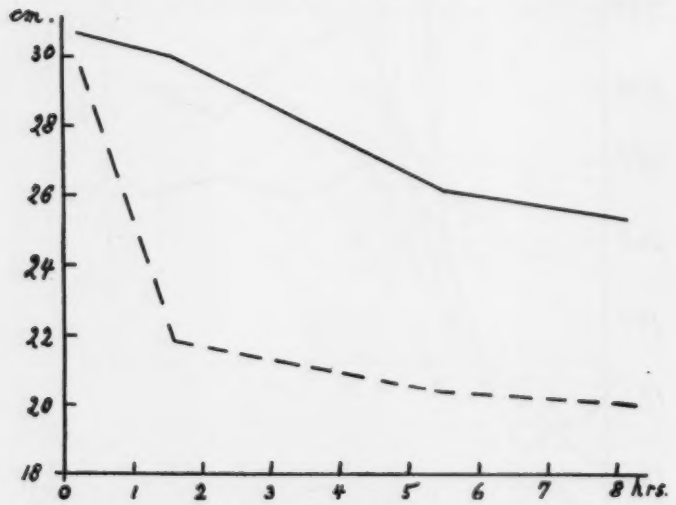

FIG. 3. The course of irritability (threshold) in unstimulated muscles. Ordinate: Distance between primary and secondary coil; abscissa: Time in hours.
————0.111 M NaCl + 10.2 M × 10^{-4} KCl + 8.1 M × 10^{-4} $CaCl_2$.
— — — —0.103 M NaCl + 10.2 M × 10^{-4} KCl + 64.4 M × 10^{-4} $CaCl_2$.

effect of the same solutions is studied on the irritability of the unstimulated muscle. As was to be expected, the solution with the "normal" $CaCl_2$ concentration is much more favorable to the preservation of irritability than a solution with higher $CaCl_2$ content (Fig. 3). Therefore the optimal conditions are different for the stimulated and unstimulated muscle. That is to say, *the laws of ion antagonism which lead to the discovery of the equilibrated solutions do not hold quantitatively for the stimulated muscle.* These experiments make probable an even

more general formulation that the efficiency of ions on cells depends upon their degree of permeability.

Experiments Performed with the Second Method

In order to check these results more than 100 experiments were performed with the second method. Figure 4 illustrates the course of fatigue after the muscles have been immersed in solutions of different $CaCl_2$ concentrations. The curves I and II show the fatigue curve of the two muscles when stimulated in the moist chamber. One recognizes that they behave identically. After this they were immersed

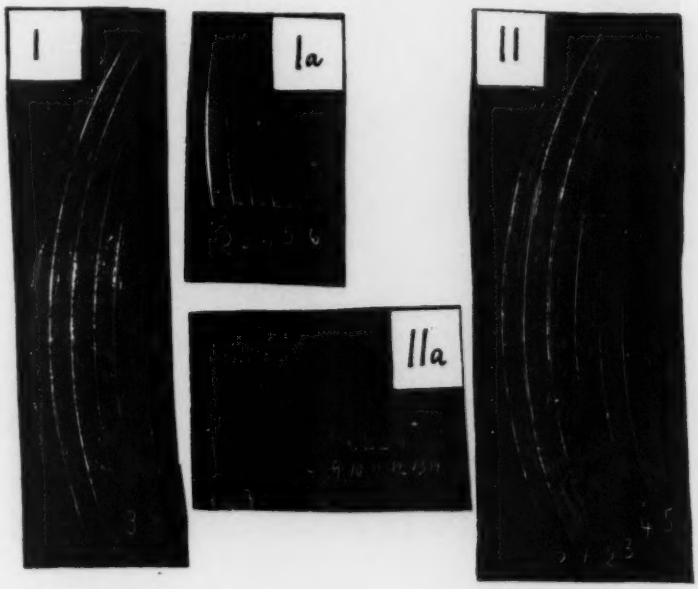

Fig. 4. Stimulation of a pair of sartorii in a moist chamber with maximal condenser discharges 90 times per minute. Then muscle I is immersed in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 8.1 M $\times 10^{-4}$ $CaCl_2$ (control fluid) and muscle II in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 81 M $\times 10^{-4}$ $CaCl_2$ for nine minutes. Thereafter stimulation in a moist chamber as before (curve Ia, IIa respectively). The numbers at the curves indicate the time in minutes after the beginning of stimulation.

for nine minutes in solutions of NaCl + KCL + $CaCl_2$ which were alike save for the $CaCl_2$ concentration. This was 8.1 M $\times 10^{-4}$ in the first case and 81 M $\times 10^{-4}$ in the latter one. Finally they were trans-

ferred again into the moist chamber and stimulated in the same way as in the first series. The experiments yielded the curves I*a* and II*a* and prove that the solution rich in $CaCl_2$ preserved the contractility of the muscle much better than that with the lower $CaCl_2$ concentration. The height of contraction which was observed in the first muscle (treated with 8.1 M $\times$ 10^{-4} $CaCl_2$) after five minutes was the same as

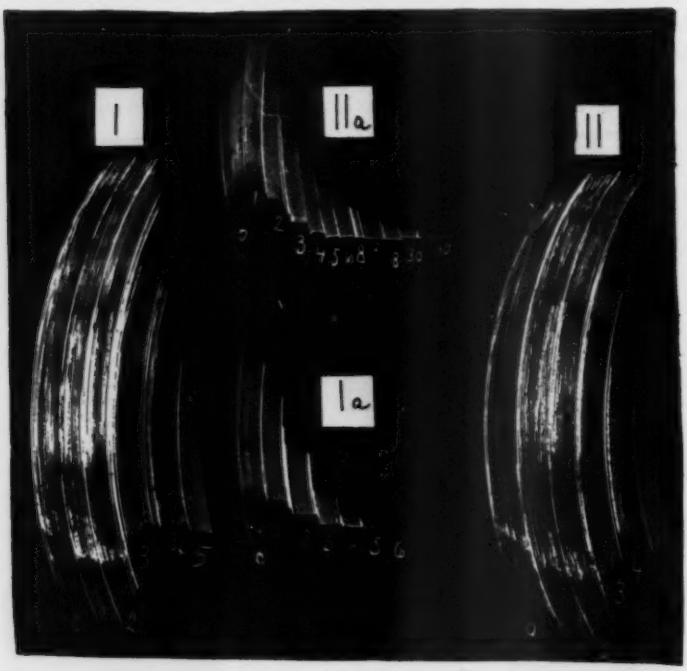

FIG. 5. Procedure as in Fig. 4. Muscle I immersed in control fluid; muscle II in 0.111 M NaCl + 10.2 M $\times$ 10^{-4} KCl + 40.5 M $\times$ 10^{-4} $CaCl_2$.

in the second muscle (treated with 81 M $\times$ 10^{-4} $CaCl_2$) after fourteen minutes.

Another example is reproduced in Fig. 5. It is of particular interest because in the first series of contractions the second muscle shows spontaneously a greater fatigability than the first one. Nevertheless, the treatment of the muscle with the solution containing a higher $CaCl_2$ concentration overcompensated the deficiency, since this muscle remains contractile for 40 minutes and the control muscle for only five

minutes. In a group of experiments the solution with the higher $CaCl_2$ concentration contained the same NaCl concentration as the other one so that the osmotic pressure of the first solution was slightly higher, while in a second group the NaCl concentration was a little lower in order to keep constant the osmotic pressure in spite of the differences in $CaCl_2$ concentration. In both groups the solution rich in $CaCl_2$

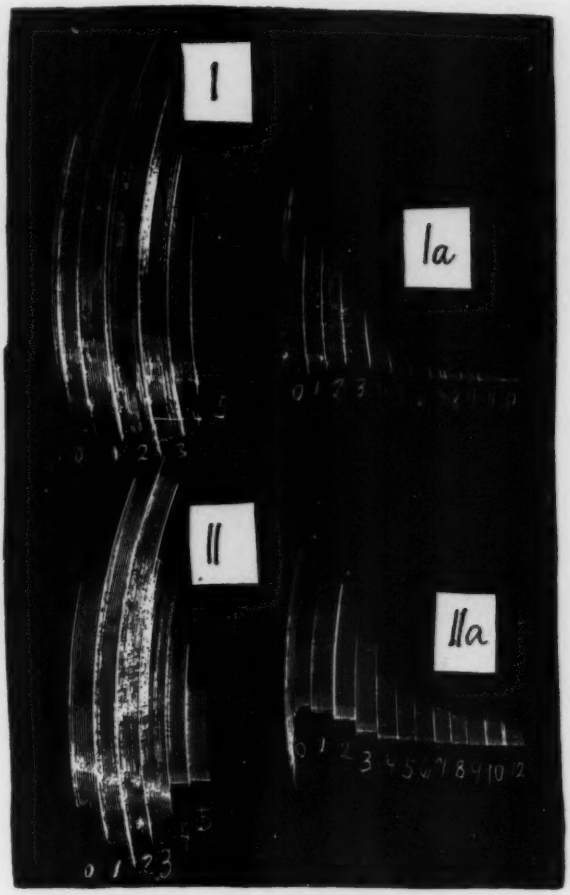

FIG. 6. Procedure as in Fig. 4. Muscle I (Fig. 1 and 1*a*) immersed in control fluid; muscle II in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 40.5 M $\times 10^{-4}$ $CaCl_2$ (Fig. II and II*a*).

delayed the fatigue, and this result was also obtained if by addition of $NaHCO_3$ the salt solution were completed to a typical Ringer's solution. Figure 6 shows this in a very striking way because, in spite of the weaker contractions of the second muscle, the fatigue is delayed after the muscle has been immersed in a Ringer's solution with five times its normal $CaCl_2$ concentration.

FIG. 7. Procedure as in Fig. 4. Muscle I in 0.111 M NaCl + 10.2 M $\times 10^{-4}$ KCl + 81 M $\times 10^{-4}$ $CaCl_2$ (1 and 1*a*); muscle II in control fluid (II and II*a*).

The favorable effect of the solution with higher $CaCl_2$ concentration in delaying fatigue may be illustrated by Table I, in which the time of the preservation of contractility was determined for each pair of

muscles after they had been immersed in salt solutions with different $CaCl_2$ concentrations.

TABLE I

The Dependence of the Duration of Contractility upon the $CaCl_2$ Concentration

After a previous period of stimulation for five minutes, the muscles were immersed in 0.111M NaCl + 0.00102M KCl + different $CaCl_2$ concentrations as indicated in this table. After this, stimulation was continued in a moist chamber.

No.	$CaCl_2$ Concentration	Duration	$CaCl_2$ Concentration	Duration
		minutes		minutes
1	$8.1M \times 10^{-4}$	5	$40.5M \times 10^{-4}$	> 17
2	"	5	$40.5M \times 10^{-4}$	> 40
3	"	6	$81M \times 10^{-4}$	> 15
4	"	4	$81M \times 10^{-4}$	> 9
5	"	4	$81M \times 10^{-4}$	46
6	"	6	$81M \times 10^{-4}$	20

The experiments with the second method reveal still another fact which seems to be of interest for the problem of the tonus of skeletal muscle. The curves of Fig. 5 in particular show that after the inter-

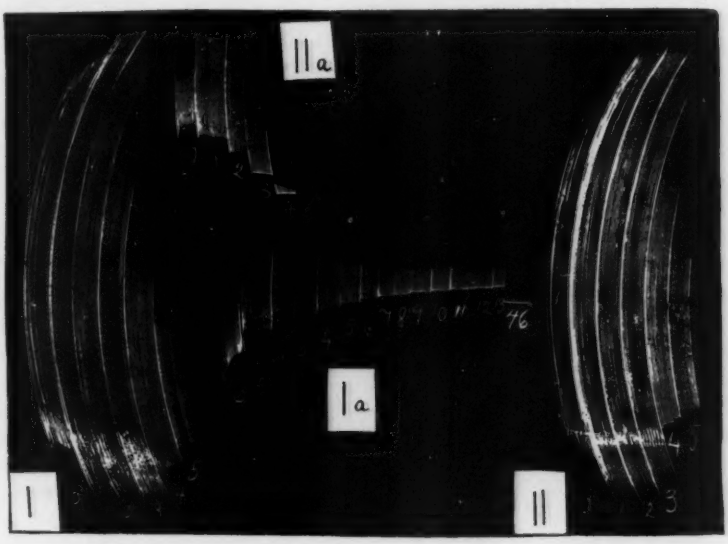

FIG. 8. Procedure as in Fig. 4. Solutions as in Fig. 7.

val during which the muscles have been immersed in salt solutions the stimulation leads to a contracture which is slightly stronger in the Ca-muscle. Furthermore, the diminution of this contracture is delayed in the Ca-muscle.[4] In this experiment the contracture still increased one minute after the beginning of stimulation while in the control muscle it diminished. This behavior is even more pronounced in the experiment illustrated by Fig. 8, in which the Ca-muscle showed an increase in tonus during almost the entire period of stimulation, although the control muscle showed a rapid loss of tonus during this time. The tonus effect of calcium becomes apparent when the $CaCl_2$ concentration is 81 M $\times$ 10^{-4} or higher. In reference to the delay of fatigue the muscles show an unequal behavior. In most cases the fatigue was delayed in spite of the increase in tonus, but in some cases (compare Fig. 7) the tonus increased so rapidly that it led to an acceleration of fatigue.

The behavior of the stimulated and unstimulated muscle in solutions containing different $CaCl_2$ concentrations was compared and it was concluded that stimulation entirely changed the reactivity of the muscle to this particular ion. This fact was explained by the increase in permeability of the muscle. The observations on the effects of calcium ions on the tonus of skeletal muscle seem to support this view since Neuschlosz (1922) observed that the immersion of the *resting* muscle in Ringer's solution with increased $CaCl_2$ concentration lead always to a *decrease* in tonus. It is probable that the muscle brought into a state of high permeability by previous stimulation takes up $CaCl_2$ in a greater amount than the resting muscle and this leads to entirely different phenomena. It may be emphasized that about the same concentrations were used in Neuschlosz' experiments and our own. If the interpretation is correct it is to be expected that far greater concentrations of $CaCl_2$ would be able to increase the tonus in the resting muscle. In fact it was found by Guenther (1905) that $CaCl_2$ in one per cent solution leads to an increase in tonus which occurs after a latent period of several hours. Probably the irritability of the muscle had faded under these conditions. In contrast to that, the experiments described above show that $CaCl_2$ may immediately raise the tonus of the irritable muscle provided that the permeability had been increased by previous stimulation. Therefore not only by K as Neuschlosz found, but also by Ca the tonus of muscle can be increased without decrease of irritability.

Leaving aside the effects of $CaCl_2$ on the tonus of muscle, the ex-

[4] For the sake of brevity the expression Ca-muscle is used for the muscle which has been immersed in the solution containing the higher $CaCl_2$ concentration.

periments lead to the conclusion that increase in $CaCl_2$ concentration through a very wide range reduces the higher degree of permeability produced by stimulation, and therefore delays fatigue. Further experiments dealt with the question whether the Ca effect is specific or whether it is replaceable by other cations. The effects of Sr, Mg and Ba were examined.

The experiments showed that *none of the alkali earths can replace calcium.* Rather small concentrations of Sr, Ba and Mg had only a slight effect if any, and greater concentrations accelerated fatigue. $MgCl_2$ seemed to have the most harmful effect, since it reduced the life duration of the muscle which had been previously immersed in a solution of $NaCl + KCl + MgCl_2 + NaHCO_3$, while the effect of Sr and Ba was mostly limited to a decrease in the height of concentration. The concentrations used are given in Table II.

TABLE II

Each solution ordinarily contains beside the salts with bivalent cations 0.111M NaCl + 10.2M × 10^{-4} KCl + 12.5M × 10^{-4} $NaHCO_3$.

	Concentration	Effect on Fatigue
Mg	15.4M × 10^{-4}	—
	23.1M × 10^{-4}	—
	30.8M × 10^{-4}	—
Ba	23.9M × 10^{-4}	—
	31.8M × 10^{-4}	—
Sr	16.7M × 10^{-4}	—
	32.5M × 10^{-4}	—
	40.7M × 10^{-4}	—
	81.4M × 10^{-4}	—

In another series one muscle was immersed in a solution of $NaCl + KCl + CaCl_2 + NaHCO_3$, the composition of which was identical with that used for the control muscle save for the addition of $SrCl_2$, $BaCl_2$ or $MgCl_2$. In these experiments it was found that all three cations were able to delay the fatigue of the muscle but the effect was much less than in the experiments described above in which an excess of $CaCl_2$ was studied. Furthermore, it was found that the favorable effect was limited to a very small range of concentrations. When this was surpassed the fatigue was accelerated. The quantitative data are reproduced in Table III.

TABLE III

The Effect of Sr, Ba and Mg on the course of fatigue if added to 0.111M NaCl $+10.2M \times 10^{-4}$ KCl $+ 8.1M \times 10^{-4}CaCl_2 + 12.5M \times 10^{-4}$ $NaHCO_3$.

	Concentration	Effect on Fatigue *
Sr	$32.5M \times 10^{-4}$	+
	$40.7M \times 10^{-4}$	+
Ba	$19.8M \times 10^{-4}$	+
	$23.9M \times 10^{-4}$	+
	$31.8M \times 10^{-4}$	−
Mg	$15.4M \times 10^{-4}$	+
	$19.2M \times 10^{-4}$	±
	$30.8M \times 10^{-4}$	−

* + indicates delay in fatigue; −, acceleration in fatigue; ±, no change.

DISCUSSION

The experiments described in this paper prove that the laws of ion-antagonism differ quantitatively in the resting and the stimulated muscle. The increase in permeability in the latter leads rapidly to fatigue and this can be delayed when an excess of Ca is added which reduces permeability. On the other hand, a Ringer's solution containing an excess of $CaCl_2$ is unfavorable for the preservation of irritability in *resting* muscle. The fundamental difference in the behavior of resting and stimulated muscle in reference to ions is further illustrated by experiments in which the effect of other bivalent cations was studied. It was found that Sr, Mg or Ba added to Ringer's solution with normal $CaCl_2$ concentration exerted a favorable influence on the stimulated muscle, that is, the fatigue was delayed. But the same solutions were unable to preserve the irritability of the unstimulated muscle as well as Ringer's solution. The experiments permit one to conclude that *the composition of the optimal salt solution is not a definite one for a certain type of cell but is dependent upon its degree of permeability.*

This implies the fact that the efficiency of ions differs quantitatively if applied to a cell in a state of low or high permeability. The experiments on the tonus effect of calcium described above show the correctness of this conclusion. The increase in tonus observed in the stimulated muscle begins immediately after stimulation, while, according to Guenther, it requires a Ca concentration ten times higher and several hours in resting muscle.

The experiments throw an interesting light upon the specificity of ion antagonism.[5] They indicate that, in contrast to the ion antagon-

[5] Compare Gellhorn, 1924 and 1926.

ism between K and bivalent cations in muscle in which numerous cations are of great antagonistic efficiency, calcium cannot be replaced by any other bivalent cation in its effect of delaying fatigue. This is astonishing because the suppression of the K contracture by bivalent cations is based upon their power to reduce permeability (Gellhorn, 1928). The writer found recently in experiments on vital staining in sea urchin eggs that the permeability of the cell to stains could be prevented by Ca but not by Mg, Sr or Ba (1931). But the explanation of the specificity seems to be quite different in sea urchin eggs and muscle, since in the former Sr, Ba and Mg were unable to decrease permeability, while in muscle permeability is decreased. This is not only indicated by observations on K-contracture but also by the fatigue experiments of this paper, since it was shown that addition of Sr, Ba or Mg to Ringer's solution containing $CaCl_2$ was effective in reducing fatigue. The reason why Ca cannot be entirely replaced by Sr, Ba and Mg seems to lie in the fact that Ca is probably the only ion which diminishes the increase in permeability of muscle in a completely reversible manner. However, when coarser changes in the surface of the cell are concerned, such as are produced by K, several bivalent cations act antagonistically. Fewer of these ions are effective if irritability is concerned, as in paralysis of muscle (Höber, 1917), than in the case of K-contracture, which is rather independent of irritability. In the former case Ca is the most antagonistic ion, in the latter case the heavy metals (Gellhorn), because here the greater the power in reducing permeability, the more efficiently is the contracture suppressed. The impossibility of fine reversible changes in permeability, which are necessary for the preservation of irritability, is not concerned here. The intermediate case, the restoration of irritability after K-paralysis, is influenced in a somewhat intermediate manner: calcium is most effective, but other bivalent cations are effective to a lesser degree. The importance of calcium for the preservation of irritability and contractility is apparent. It is conceivable that the delay of fatigue requires a still finer adjustment than the partial restoration of irritability after K-paralysis. This may be the reason for the specificity of the calcium effect in the fatigue experiments of this paper.

Summary

1. The fatigue of the sartorius muscle of the frog can be delayed by an excess of calcium chloride in Ringer's solution, although this solution does not preserve irritability of resting muscle as well as Ringer's solution. Therefore, in contrast to the view generally accepted today, *the quantitative composition of the optimal solution depends upon the permeability of the cell.*

2. This conclusion implies a quantitative difference in the efficiency of ions in relation to permeability. This is illustrated by observations on the increase of muscle tonus in solutions with high $CaCl_2$ content. The change in tonus takes place in irritable muscle.

3. The effect of calcium on fatigue is specific. Neither Sr, Ba nor Mg can replace it. But these cations can delay the fatigue, although to a lesser extent than calcium, when added to Ringer's solution containing 8.1 M $\times$ 10^{-4} $CaCl_2$. The cause of this specificity is discussed.

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THE RECOVERY CONTRACTURE IN MUSCLE; A NEW GENERAL SALT EFFECT[1]

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In a study on permeability and fatigue in muscle and its bearing on the problem of ion antagonism, the method used in a series of experiments was as follows. The sartorius muscle was stimulated for five minutes with condenser discharges about 60–90 times per minute. Thereafter the muscle was allowed to rest for at least five minutes in Ringer's solution with normal or increased Ca content and then a new series of stimulations of the same frequency was begun. The muscle was in a moist chamber when stimulated. Under these conditions a peculiar phenomenon was observed. In the beginning of the second stimulation series the muscle showed a contracture which became less during the continuation of the stimulations and finally disappeared more or less completely. The phenomenon is fundamentally different from the fatigue contracture in muscle which increases with the continuation of stimulations. The experiments described below serve to analyse the phenomenon.

Method

The experiments were carried out on m. sartorius and biceps of *Rana esculenta*. The carefully prepared muscles were suspended between platinum electrodes and stimulated by means of the apparatus of Scheminsky, which allowed one to use condenser discharges over a wide range of frequencies. The strength of the stimulus was varied by a parallel resistance; there was also a resistance of 10,000 ohms in series in order to make very slight changes in the conductivity of the muscle ineffective. The stimuli were either just maximal or submaximal. The muscles were loaded with 2 g; the magnification of the isotonic lever was six fold. The experiments were performed from October 1930 to January 1931.

Results

I. The Basic Phenomenon

A typical example is reproduced in Fig. 1. In this experiment the sartorius was stimulated 90 times per minute with a condenser of 0.5 mf. Between the periods of stimulation the muscle recovered in a

[1] Aided by a grant from the research fund of the University of Oregon.

well-aerated Ringer's solution. Each period of stimulation and recovery lasted for five minutes. One recognizes from Fig. 1 that the curves *b–d* show in the beginning contractures which decrease while stimulation is being continued. There is a regular decrease in the

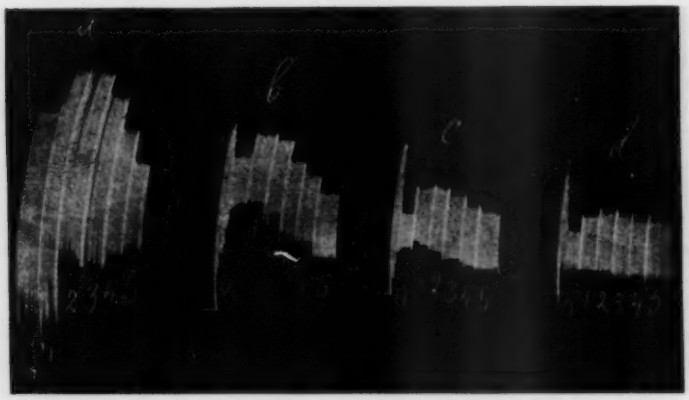

FIG. 1. Four stimulation periods on sartorius. Condenser discharges (0.5 mf.) 90 times per minute, in a moist chamber. Muscle is in Ringer's solution during recovery period. The conditions in all succeeding experiments are the same unless otherwise specified

height of contracture from *b* to *d*.[2] This makes it plain that the phenomenon in question is basically different from the fatigue contracture since (1) fatigue contracture increases with the continuation of stimulation and (2) in spite of a slight decrease in the height of contractions from *b* to *d*, *i.e.*, an increase in fatigue, the contracture becomes less. This peculiar behavior of the contracture makes it rather probable that it might be due to a recovery and not to a fatigue process. As to the height of contractions, it is characteristic that although the contractions decrease in the very beginning of the contracture until the maximum of the contracture has been reached, hereafter a remarkable increase in the height of contractions is accompanied by a decrease in contracture.

There are still other facts which support the hypothesis that the phenomenon is a recovery contracture. It is known that with increasing fatigue the duration of the contraction increases. The detailed study has shown that the change in duration chiefly concerns relaxation. At first one may see that an elastic vibration ends the

[2] In the text and in the figures the different stimulation periods are designated as *a*, *b*, *c*, *d*. The first which can show recovery contracture is the *b* curve.

period of relaxation. With increasing fatigue the elastic vibration disappears and a slow contraction (designated as "Funke's Nose") is apparent in the downstroke of the contraction. The more fatigue progresses the earlier in the downstroke appears this slight contraction. Since the relaxation period of the "Funke's Nose" is much longer than in a normal contraction the duration of the contraction increases in correspondence with the earlier appearance of the "nose." The registration of the recovery contracture on a faster revolving drum revealed the interesting fact that the changes described as characteristic for fatigue (Funke, Wachholder) also occur during the recovery contracture but in the *reverse order* as is shown in Fig. 2, in which tracings obtained at intervals of one minute are recorded.

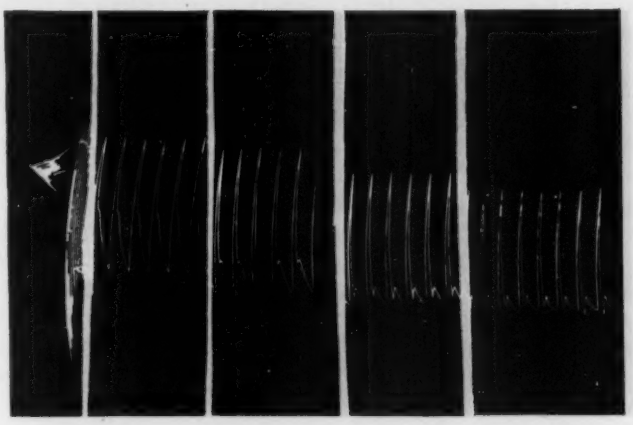

FIG. 2. *b*-curve on a faster moving drum.

It was stated above that the recovery contracture decreases with increasing number of alternating stimulation and recovery periods as is apparent if the recovery contracture in Fig. 1, *b* is compared with 1, *c* or 1, *d*. Experiments on a fast revolving drum revealed that the changes in shape and duration of the contraction correspond to simultaneous changes in the size of contracture. In an experiment, for instance, which was carried out in a similar manner as that reproduced in Fig. 1, it was found that the disappearence of the "Funke's Nose" required one minute in experiment *d*, four minutes in experiment *c* and more than five minutes in experiment *b*. In other words, the recovery phenomenon judged in its intensity according to the height of contracture and to the time during which the "Funke's Nose" appears decreases the

more the fatigue increases. That is, of course, to be expected, since recovery is more marked in a slightly fatigued muscle than in one which has been very much fatigued. Thus the phenomenon is undoubtedly a recovery contracture and the question arises as to what factors are responsible for its occurrence.

II. The Influence of Strength, Frequency, and Duration of Stimuli on the Occurrence of Recovery Contracture

Systematic studies showed that the recovery contracture is rather independent of the strength of the stimuli since it occurs in experiments with submaximal as well as maximal condenser discharges. This makes it plain that the contracture is different from Tiegel's contracture, which requires the application of supermaximal currents. However, the frequency of stimulation had a marked influence. Experiments were carried on in which the frequency of stimuli in the first and second periods varied independently over a range of from 30 to 150 per minute. It was apparent that at least 45 stimulations were required in the first period to bring about the contracture in the second, provided that during this period the frequency was at least one hundred. Under these conditions, the higher the frequency employed in the second period, the more marked the contracture. But this is true only up to a frequency of 120 per minute, since beyond this value the contracture dropped slightly. It is easily understandable that stimulations below 45 per minute during the first period did not produce a recovery contracture in the second even if very high frequencies were used, since under these conditions in the first period no fatigue was observed. Therefore, a recovery phenomenon must fail to appear.

In general it may be said that the higher the frequencies used in both periods, the more marked the recovery. But even after a first period of high frequency the contracture does not occur unless the mus-

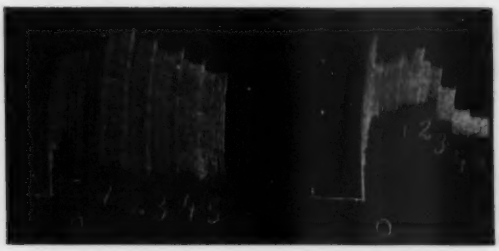

Fig. 3. *b*-curve in two sartorii of the same frog. Frequency of stimulation in the first *b*-curve 60 per minute; in the second, 120 per minute.

cle is stimulated at least 45 times per minute in the second period. The strength of the recovery contracture is not only apparent in its height but also in the changes in the shape and duration of the contraction. This may be illustrated by Figs. 3 and 4. In Fig. 3 the recovery contracture of two sartorii of the same frog are reproduced. The frequency during the first period was the same in both cases (90 per min-

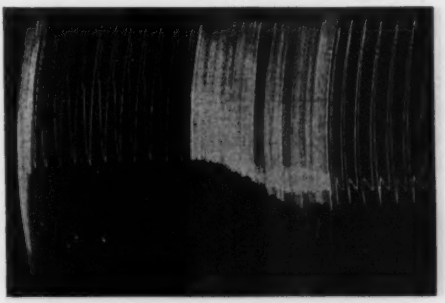

FIG. 4. *b*-curve. Frequency in *a*-curve was 60; in *b*, 90 per minute.

ute), while in the second period the first muscle was stimulated 60 times and the second muscle 120 times per minute. Fig. 4 shows that the "Funke's Nose" disappears in the downstroke of the contraction within one minute, frequency of stimulation being 60 in the first and

FIG. 5. *b*-curve. The two sartorii were stimulated in the *a* period with a condenser 0.1 mf.; in the *b* period of the first muscle a condenser of 2.0 mf. was used, in that of the second a condenser of 0.1 mf.

90 in the second period, while if during both periods the muscle is stimulated 90 times per minute, it takes much longer for the duration of contraction to become normal. (Compare Fig. 2.)

The duration of the stimulus also seems to affect the recovery

contracture. In Fig. 5 the recovery contracture of two sartorii of the same frog is reproduced from an experiment in which strength and frequency of the stimulus were identical, but the duration of the stimulus was in the first muscle 70σ and in the second 20σ. One recognizes in Fig. 5 that increasing duration of the stimulus increases recovery contracture. But even with very short currents the phenomenon can be observed, since recovery contracture is obtained in sartorius after application of submaximal induction currents. It also shows under these conditions the characteristic features mentioned above which concern the occurrence of the "Funke's Nose" and the duration of the single contraction.

III. The Influence of Salts and Non-electrolytes on the Occurrence of Recovery Contracture

This paragraph deals with the problem of the physico-chemical factors which determine the occurrence of the recovery contracture. In the first group of experiments one muscle recovered in the moist chamber while the other muscle of the same frog recovered in Ringer's solution as in the previously described experiments. In order to get

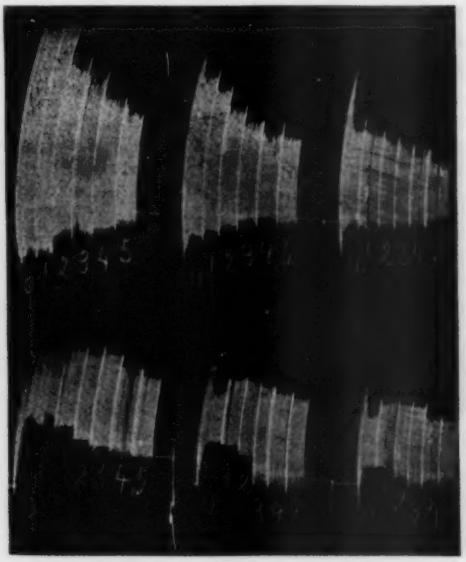

Fig. 6. *b–d* curves of two sartorii of the same frog. Upper tracing: recovery period in moist chamber. Lower tracing: recovery period in Ringer's solution.

distinct results it is advisable to avoid soaking the muscle in Ringer's solution for a long time when it is afterwards to be in the moist chamber during the recovery period.

Dulière and Horton observed recently that a muscle which is brought into a moist chamber without having been in contact with Ringer's solution lost its irritability. The observations of these authors were confirmed and therefore the muscle was immersed in Ringer's solution for two minutes before being placed in the moist chamber. Under these conditions the irritability of the muscle did not change and the very striking result was regularly obtained that the muscle which recovered in a moist chamber did not show recovery contracture, while the muscle immersed in Ringer's solution during recovery period always showed the contracture. Figure 6 gives an illustration of the

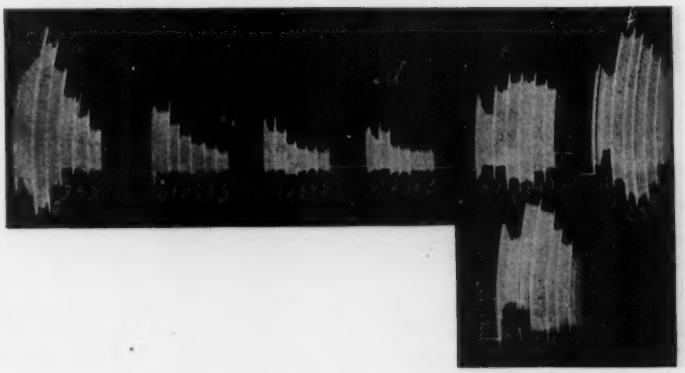

FIG. 7. *a–g* curves of sartorius. Between *a* and *d* period, muscle in moist chamber; between *d* and *g*, in Ringer's solution during recovery.

typical results for three periods (*b-d*). While the "Ringer" muscle undergoes the typical recovery contracture, decreasing more and more with each stimulation period, the "air" muscle does not show any contracture.[3] The change in the shape and duration of the contractions is also restricted to the muscle which recovered in Ringer's solution and showed recovery contracture.

Experiments of this type were performed in great number without a failure. It is interesting to note that, as Fig. 7 indicates, alternating conditions bring about alternate presence and absence of the contrac-

[3] For the sake of brevity the muscle which remained in Ringer's during the recovery period is referred to as the "Ringer muscle," that which remained in the moist chamber, the "air muscle."

ture of the muscle. In this experiment the muscle remained during the first three recovery periods in a moist chamber and therefore did not show a recovery contracture; after the fourth stimulation period it always remained in Ringer's solution during the recovery period. The effect is very significant. The long period in air influenced the behavior of the muscle so that one recovery period in Ringer's did not immediately bring about the contracture. Therefore it is not noticeable in experiment *e*, but it becomes apparent in *f* and still more in *g*.

The time during which the muscle was kept in Ringer's solution before the experiment was started is not immaterial to its reaction in reference to the recovery contracture. Thus it was found that soaking the muscle for 24 hours in Ringer's solution increases its tendency to react with recovery contracture even if the muscle is kept in a moist chamber during the recovery period. This may be illustrated by Fig. 8. The first curve showed a typical although very slight recovery

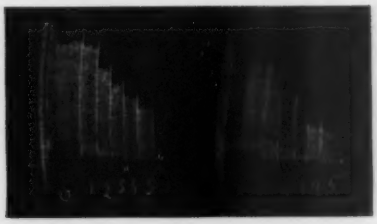

FIG. 8. Both sartorii soaked for 24 hours in Ringer's solution. First muscle immediately thereafter stimulated as in Fig. 1, the second muscle remained one hour in moist chamber before stimulation. Muscles in moist chamber during recovery period. *b*-curves reproduced.

contracture. But the contracture was still smaller in the second sartorius of the same frog, which was held in a moist chamber for one hour before starting the stimulation. It was, of course, also soaked for 24 hours in Ringer's solution. Besides, the second muscle shows but a slight decrease in the elastic vibrations during contracture while the first one did not show them at all. This indicates, as was proven by registering on a fast revolving kymograph, that in the first case the contracture was accompanied by the occurrence of the "Funke's nose," which did not occur in the second case. Summing up these observations, it may be said that recovery contracture occurs if the muscle is kept in Ringer's solution during the recovery period and it disappears if the muscle is in a moist chamber during that period. The effects are reversible. The soaking of the muscle in Ringer's solution furthers the recovery contracture.

Seeking for an explanation of this peculiar phenomenon it seemed rather probable that the presence or absence of the contracture might be due to differences in ion concentrations at the surface layer of muscle, since the efficiency of ions to produce contractures and influence the "tonus" of muscles is known (Neuschlosz, Gellhorn). But experiments did not prove the correctness of this view. The recovery contracture occurs not only if the muscle is kept in Ringer's solution but also if the composition of that solution is much altered. An increase in the K or Ca concentration to ten times its original value did not noticeably influence the strength of the recovery contracture. The phenomenon was also observed in isotonic NaCl, Na_2SO_4 and LiCl solutions. If the K or Ca content of the muscle were responsible for the phenomenon in question, changes in concentration of these ions should be effective. Therefore it was thought that one might have to do here with a general salt effect as was first observed by Loeb (for further references compare Gellhorn, 1926 and 1929, pp. 153–163). In this case the contracture should be diminished or should not occur at all if the muscle were placed in a non-electrolyte solution during the recovery period. In fact, the experiments carried out with glucose, sucrose and urea either alone, in isotonic solution or replacing different

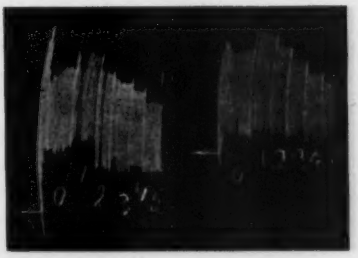

FIG. 9. *b*-curves. (m. sartorius.) Muscle 1 in Ringer's, muscle 2 in 3 cc. Ringer's + 17 cc. isotonic urea during recovery period.

parts of Ringer's solution, yielded the expected results. Figures 9 and 10 show a typical experiment on sartorius and biceps. In Fig. 9 the recovery contracture was much decreased if the muscle remained in a solution consisting of Ringer's + urea during the recovery period. In Fig. 10 it is shown that recovery contracture is suppressed completely if the muscle is immersed in isotonic sucrose solution during the recovery period.[4]

[4] In some of the experiments it was observed that the sartorious muscle showed a small contracture when immersed in isotonic non-electrolyte solutions. Therefore in most cases mixtures of Ringer's and non-electrolyte solutions were employed which did not produce a contracture during the recovery period.

As mentioned above, the experiments were carried out on the sartorius and biceps of *Rana esculenta*. The results were the same in both muscles. This seems of interest in respect to observations of Sommerkamp, who found that some groups of muscles react more to reagents which produce contractures, especially to acetylcholin, than others. This fact was explained by structural differences because the

FIG. 10. *b*-curves. (m. biceps.) Muscle 1 in Ringer's, muscle 2 in isotonic sucrose during recovery period.

content of "tonusfibers" is different in different muscles. The observations of this paper seem to indicate that in the recovery contracture only the ordinary muscle fibers are involved since biceps and sartorius behaved rather alike in spite of the differences observed by Sommerkamp in experiments with acetylcholin.

As to the explanation of the recovery contracture, several facts support the assumption that the condition of the surface layer of the muscle is the determining factor for its appearance. This is not meant in the sense of the theory of Botazzi, who believes that sarcoplasm is responsible for "tonic" reactions, but there are a number of observations which show more or less the dependence of contractions and contractures upon the surface layer of the muscle cell and its permeability. It will be recalled that fatigue in muscle can be delayed by an excess of calcium in Ringer's solution (Gellhorn), and furthermore, that K and SCN contractures depend entirely on permeability (Gellhorn).

The importance of the behavior of the surface of muscle for the recovery contracture is expressed by two groups of observations: 1. It was shown that the recovery contracture is increased by the application of condenser discharges with relatively long duration of discharge. As was recently proved by v. Gulacsy and Heller such discharges bring about marked polarisation phenomena on the surface layer of muscle. 2. Experiments were described demonstrating that the recovery contracture was diminished or suppressed in the presence of non-electrolytes. This shows that a general salt effect is present as in the experiments of Loeb. It still remains a question as to whether the same process underlies the salt effect in Loeb's and in our own obser-

vations. But the experiments seem to point out that the conditions of the colloids in the surface layer of the cells are different when recovery contracture occurs and when it is prevented.

Summary

If the sartorius or biceps of *Rana esculenta* is stimulated with condenser discharges in periods of five minutes with a frequency varying between 45 and 150 per minute and stimulation periods alternate regularly with recovery periods of the same duration, a recovery contracture is brought about in the beginning of the second and each following stimulation periods provided the muscle is allowed to recover in an aerated salt solution. Wide changes in the K and Ca concentration of the Ringer's solution are without influence. The contracture also occurs when isotonic solutions of Na or Li salts are employed. The phenomenon is suppressed by replacing the salt with a non-electrolyte. The following observations are in favor of the assumption that the contracture is a recovery contracture:

1. The phenomenon does not occur if the frequency is too low to produce even a slight fatigue.

2. It decreases from the second to the fourth stimulation period, *i.e.*, with increasing fatigue.

3. The changes in the shape of the contractions (Funke's Nose) during the recovery contracture show a temporal sequence just the opposite of that in fatigue.

It is assumed that changes in the surface layer of the muscle cell bring about the recovery contracture. In favor of this hypothesis are the facts:

1. The recovery contracture is increased with increasing duration of the electrical discharges which were used to stimulate the muscle.

2. Non-electrolytes which suppress the phenomenon bring about changes in the surface layer of cells.

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NOTE ON DR. JAN HIRSCHLER'S PAPER ON SPINDLE BRIDGES, AND THE *DE NOVO* ORIGIN OF MOTH SPERMATOCYTE GOLGI BODIES

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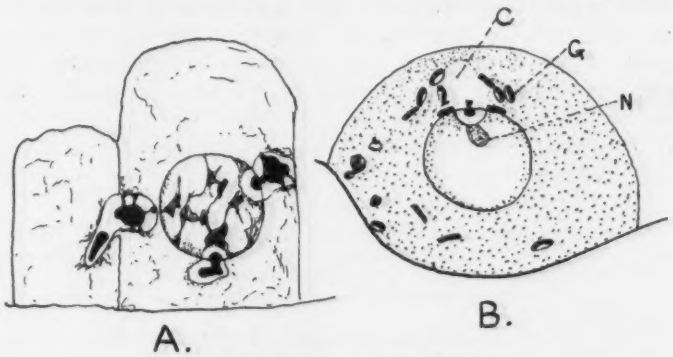
INTRODUCTION

In a recent paper Jan Hirschler (1929) has described fully in *Macrothylacia rubi* L., a remarkable relationship between certain cytoplasmic elements, and the nucleus. He states that he has found this in other genera of Lepidoptera, namely, *Phalera*, *Dasychira*, and *Dendrolimus*.

Hirschler's technique consisted in Zenker fixation followed by iron alum staining, and chrome-osmium acid fuchsin for Golgi bodies. Apparently the Zenker method shows no cytoplasmic inclusions but does show very remarkably a connection between hemispherical nucleoli and spindle bridges, such as does not appear to have been described before. In text figures A and B are reproductions of two



TEXT FIGS. A AND B
After Jan Hirschler (1929*a*)

A. Zenker fixed cells showing spindle bridges and attachments to nucleoli ("tres analogues aux hétérochromosomes d'autres Insectes").

B. Osmic Golgi preparation, showing Golgi bodies (*G*) originating around a spindle bridge space (*C*). Ordinary nucleolus, *N*. Both figures from *Macrothylacia rubi*.

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of Hirschler's figures illustrating in the first, Zenker hæmatoxylin prepared cells with two curious hemispherical nucleoli at the nuclear membrane, each connected with an extension into the cytoplasm, described by Hirschler as "résidus fusoriaux." The nucleoli he claims to be analogous with the heterochromosomes of other insects. Prepared by an osmic Golgi method, one gets the picture shown in text figure B. The hemispherical nucleolus with its pale halo, has a small object protruding from it into the cytoplasm; a normal or ordinary nucleolus (*N*) adheres to the hemispherical one. Now, just in this region there is a pale zone (*C*) surrounded by Golgi bodies. Hirschler says, "Nous considerons cette zone comme le résidu fusorial et nous pensons que, sous l'influence de la substance nucléaire qu'elle renferme et qui touche au cytoplasme, il se forme dans celui-ci, par précipitation des éléments de l'appareil de Golgi qui peuvent ensuite perdre contact avec le résidu fusorial et se transporter en d'autres régions de la cellule."

In a previous paper (Gatenby, 1931*a*) the present writer has referred to the same idea of Hirschler, by which a new formation of secondary Golgi bodies is supposed to take place even in the spermatids of a pentatomid. This hypothesis of Hirschler (1929*b*) has been dealt with in the previous paper, and is considered untenable so far as it applied to insect germ cells studied by the present writer.

The Material and Methods.—The material used in the present work consisted of a large series of normal, x-radiated and radium-radiated testes of *Abraxas*. No material from other moths is at present available, but a large number of lepidopterous testes from other families has been studied in recent years.

The Spindle Bridge in Abraxas.—In Fig. 2 of the plate is a group

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DESCRIPTION OF PLATE

Letters: B, bacteria. *BR*, branch of spindle bridge. *D*, dead cell. *GB*, Golgi body. *L*, living cell. *SB*, spindle bridge. *X*, dying cell. *Y*, normal-looking cells.

All *Abraxas* and, except Fig. 3, by Champy hæmatoxylin.

FIG. 1. Group of spermatocytes, some normal-looking (*Y*), one dying (*X*), as the result of x-radiation.

FIG. 2. Rosette of spermatogonia showing live and dead cells as result of gamma-radiation. Spindle bridge at *SB*. Such cells already contain Golgi bodies as in next figure.

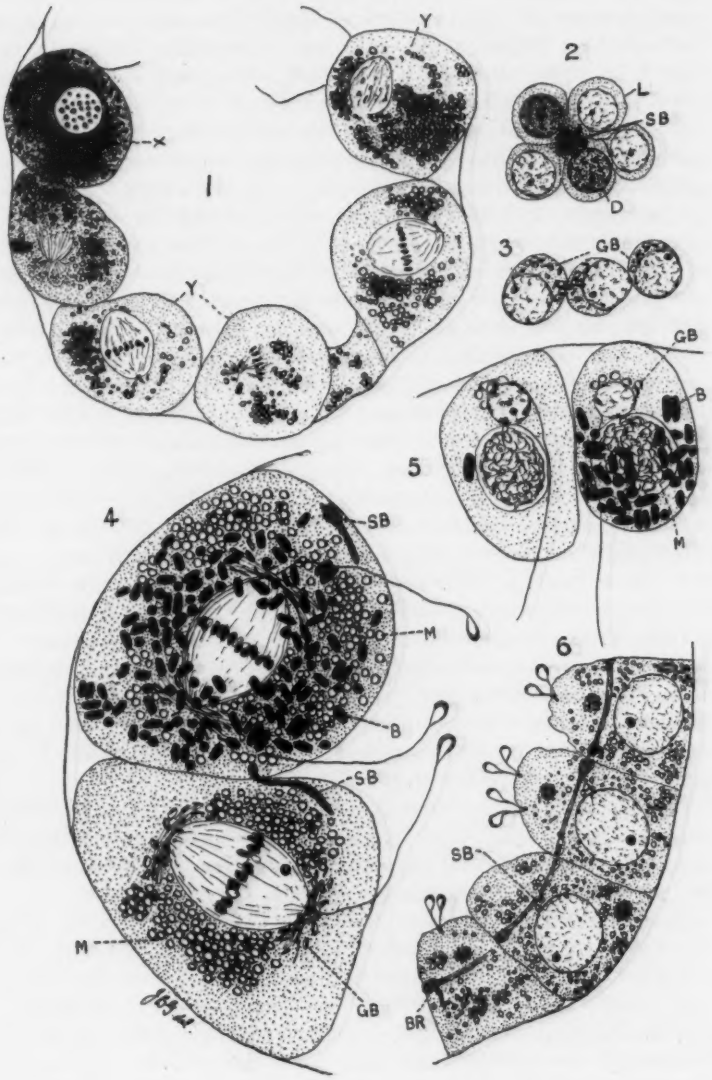
FIG. 3. Nassanow method. Rosette spermatogonia with numerous Golgi bodies. These cells are much smaller than those in text figures A and B, in which Golgi bodies are supposed to be arising *de novo*.

FIG. 4. Two spermatocytes, with metaphase spindles, showing dense bacterial infection in one, and freedom from infection of the other.

FIG. 5. Two spermatids, one badly infected, the other containing one bacterium.

FIG. 6. Group of growing spermatocytes showing spindle bridges, mitochondria and neutral red vacuoles (just above the bridges).

PLATE I



of spermatogonia connected by blackly-staining spindle bridges. When the cells hollow out to form the typical nest of growing spermatocytes, each unit is found to be connected to its neighbour by long lighter-staining bridges which pass right across the cytoplasm, in front of the nuclei, as shown in Fig. 6, *SB*. In no case has the spindle bridge been found to lie in any other region.

In spermatogonia and young spermatocytes, the spindle bridges stain sharply in chrome-osmium material which has been fixed overnight and well washed out. As the cells become larger the spindle bridges become more difficult to find under the Champy or F. W. A. hæmatoxylin treatment. It is only in special regions that the bridges are shown as clearly as in Fig. 4, at *SB*, in full grown spermatocytes.

Now in no case, in chrome-osmium hæmatoxylin preparations, could the structure depicted in text figure A be found. The spindle bridges were "solid" and never appeared to consist of two parts, inner chromophile region, and an outer canal-like structure. It was also in vain that in the chrome-osmium hæmatoxylin slides the modified heterochromosomes (hemispherical nucleolus) were looked for. In the case of the Kolatschew preparations nothing corresponding to the hemispherical nucleolus was found. The writer is certain that the ordinary nucleoli of *Abraxas* are not heterochromosomes (Gatenby, 1931*b*), and has found as in Fig. 6, that they lie in all directions in the nuclei, some towards, others away from the spindle bridge (*SB*). Very careful examination failed to reveal any side branch coming from the spindle bridge towards the ordinary nucleolus. Of course, it is quite possible that Champy's fluid and iron alum hæmatoxylin may have failed to show both the side branch and the hemispherical nucleolus. It is not thought that Jan Hirschler's figures are not correct, but it can only be stated that *Abraxas* must be very different from the forms studied by him.

Then to refer to text figure B—nothing like this has ever been found in *Abraxas* except in the beginning stages of the formation of abortive acroblasts (Gatenby, Mukerji, and Wigoder, 1929), which was a purely experimental phenomenon.

The Origin of Golgi Bodies in Moths.—Hirschler makes the remarkable statement that he believes that Golgi bodies appear in *Macrothylacia*, under the influence of nuclear substance, on the edge of the spindle-bridge shown in text figure B at *C*. Here again the present writer finds this somewhat difficult to credit. In Fig. 3 of the plate are three very small spermatogonia from rosettes; these cells already contain Golgi bodies. All the evidence which the writer could find seems to indicate that Golgi bodies are to be seen in the

smallest rosette cells in young testes, and do not originate as claimed by Hirschler. Nor do the ordinary nucleoli in such cells bear any relationship to the spindle bridges, the confines of which (Fig. 2) are clearly demonstrable.

The Function of the Spindle Bridges.—It seems certain that the function of the spindle bridges in such animals as the moths, is to keep all the cells in one nest at approximately the same stage of growth and physiological condition. For in the testis crowded with all stages of spermatogenesis, some cells lying nearer the testicular wall are better exposed to nutriment than others.

That there must be some sort of connection between the cells of a nest seems well shown by the "experiment" in Fig. 4. *Abraxas* is often infected with large bacteria which live in many of the cells of the body, and quite often in the testis. Here are two spermatocytes in first maturation division—both at the same stage, but one cell is simply crammed with bacteria, so as almost to hide the spindle. Now it appears unlikely that a cell so burdened could keep up with its neighbours unless it received active help from them. Yet such infected cells go successfully through maturation and produce spermatids as in Fig. 5, which are perfectly normal except for the bacteria. Finally, the latter come to lie on the sperm tails and are probably instrumental in spreading the infection to non-infected eggs. In testis cells, such bacteria are not symbiotic.

Radiation and Cell Nests.—Radiation by either x- or gamma-rays, provides another aspect to this question, and seems to show that the injury caused by this type of treatment cannot be made up by assistance from neighbouring cells. In Fig. 2, is a rosette of cells, which had been treated by gamma rays; the cells *D*, are dead, the cells *L*, alive. In the same way the older cells in Fig. 1 had received one dose of x-rays, and were left for some hours, yet they all differ in the manner in which they have responded; the cells *Y* are normal-looking, whereas that at *X* has been badly hit.

In radiated testes of *Abraxas* left for hours or days, the badly stricken spermatocytes drop out and degenerate, leaving gaps in the nest. The survivors go on developing, often quite normally beside the degenerate remains of their less fortunate neighbours.

Discussion

Hegner (1914) shows in his figures of *Leptinotarsa*, clavate spindle bridges, the broad ends of which impinge on the spermatogonial nuclei, but have no connection with heterochromosomes or nucleoli. In a previous paper (Gatenby, 1917) clavate spindle bridges in sperma-

togonia are drawn, but these do not quite impinge on the nuclei (1917, Pl. 25, Fig. 46, *Euchelia jacobaeae*). In the case of three young spermatocytes of *Euchelia*, there is certainly no connection between the spindle bridges and the nucleoli (1917, Pl. 25, Fig. 45). In recent preparations made by the Nassanow method, it has been found that Golgi bodies exist in the earliest spermatogonial rosettes and in these the spindle bridges certainly do not touch the nuclei, while no nucleoli other than the normal ones can be discovered. The author's previous work on *Euchelia* and *Smerinthus* is also in agreement. The spindle bridges were carefully worked out in a number of species of moths without finding anything like Jan Hirschler's text figure B, and the only conclusion possible is that these moths must differ very much from Jan Hirschler's genera.

On the comparison made by Hirschler between heterochromosomes and the peculiar hemispherical nucleoli, the author is somewhat doubtful but does not care to express a definite opinion.

Hirschler's text figure A seems too definite an arrangement to be artifically produced, and the writer is willing to believe that a trial with Zenker's fluid may show something similar in *Abraxas*. Against this, however, is the fact that Hirschler figures the same thing in text figure B, done by osmic methods, as used by the writer in his collection.

On the question of the origin of Golgi bodies from nuclear sap, the author finds himself in complete disagreement with Jan Hirschler. It will be remembered that Hirschler (1929*b*) has advanced a similar idea in the case of the pentatomid spermatid worked out by him. It is not suggested that mitochondria and Golgi bodies could not originate from nuclear material, but that in the two cases cited by Hirschler, Golgi bodies were already present, before they were supposed by him to arise by some form of condensation.

The response of cells in a nest to radiation, is peculiar and indicates that some of the cells have been affected much more than others. Nor do the unaffected units manage to save their stricken neighbours. Whatever be the exact basis of communication via spindle bridges, between the cells of a nest, it is evident that the radiation injury cannot be repaired by ordinary nutrition as is presumed to be supplied through the channels formed by spindle bridges.

Summary

The spindle bridges of *Abraxas* spermatocytes do not appear to have any relationship with the *de novo* formation of Golgi bodies. No hemispherical nucleoli analogous with the heterochromosomes of

other insects have yet been found in *Abraxas*. The Golgi bodies are present in the smallest spermatogonial rosettes in which the newly-formed spindle bridges are unrelated to the nuclei; in such cells the nucleoli have no connection with the spindle bridges. The spindle bridges, as has been generally concluded, are a means whereby sister cells in a nest are kept at the same state of nutrition. Spermatocytes very badly infected with bacteria, keep step with non-infected sister cells, this, it is concluded, by virtue of the spindle bridges. Radiated testes show nests with dying and apparently undamaged cells side by side. This type of injury appears to be something different from mere inadequacy of nutrition, such as the presence of large numbers of bacteria could bring about.

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